Comparison of the action of primary alcohols as inhibitors of a soluble and a membrane-bound cytochrome P450 hydroxylase

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Abstract

This thesis describes the development of a procedure for comparing the manner in which organic solvents inhibit biocatalysts. The research compared the effect of a series of primary alcohols on a membrane bound and a soluble cytochrome P450 hydroxylase.

The effect of alkanes and primary alcohols on the bioluminescence of luciferase (EC 1.13.12.7) was developed as the basis of a fast and stable bioassay for measuring the aqueous concentration of a primary alcohol or an alkane **(Franks & Lieb, 1984).** This was then used to determine the concentration of organic solvents in the aqueous phase of a mixture of biological membranes and water.

The bioassay was used in a study of the effect of primary alcohols on two cytochrome P450 hydroxylases. The first was a purified and soluble cytochrome P450, derived from *Bacillus megaterium* (recombinant P450 BM3 engineered for high expression in *Escherichia coli* (kindly supplied by Dr Munro at the University of Edinburgh)) and the second a membrane associated enzyme present in *Rhizopus stolonifer* (formally known as *R. nigricans*).

The effect of the solvents in the reaction mixture was characterised by their IC_{50} value, that is, the concentration of the solvent that caused a 50 % inhibition of the enzyme activity. The soluble enzyme from *B. megaterium* was found to be more strongly inhibited by short chain alcohols (IC_{50} for methanol = 0.71 M) than the enzyme from *R. stolonifer* (IC_{50} for methanol =1.63 M).

The inhibition of both enzymes by the primary alcohols becomes more extreme as their molecular weight increased, and they were almost equally inhibited by pentanol (IC_{50} =62 mM and 46 mM respectively). Hexanol inhibited the enzyme from *R. nigricans* (IC_{50} =25 mM) but was not sufficiently soluble in water (solubility limit is 50.1 μM at 25°C,Bell, 1972) to inhibit the enzyme from *B. megaterium.*

The aqueous concentration of various primary alcohols and alkanes in the presence of the cells of *R. stolonifer* suggested that at 50 % inhibition the molar concentration of the solvent in the membranes of the cells was the same for all the alcohols studied.

The log IC_{50} (the logarithm of the concentration of the alcohols that caused a 50 % inhibition of the enzyme activity) was found to be a linear function of the carbon chain length of the alcohol and decreased regularly as the chain length increased. A multiple regression analysis showed that the effect of the series of primary alcohols on the two hydroxylases studies was significantly different.

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ABBREVIATIONS and NOMENCLATURE

[x]	concentration of x
ε_λ	extinction coefficient at wavelength, λ
λ	wavelength, units of nm
°C	temperature, units of degrees Celsius
2'-AMP	adenosine-2'-monophosphate
aq	aqueous phase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
A_λ	absorbance, at a wavelength λ, in units of nm.
B. megaterium	*Bacillus megaterium*
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
BSA	bovine serum albumin
CO	carbon monoxide
dwt	dry cell weight
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
E. coli	*Escherichia coli*
FPLC	fast protein liquid chromatography
gly-gly	glycine salts (Glycyl-glycine)
g	acceleration (9.81 ms^{-1})
hr	hours
H^+	hydrogen ions (H_3O^+)
HPLC	high performance liquid chromatography
HSA	human serum albumin
IPTG	isopropyl-β-D-thiogalactopyranoside
IC_{50}	concentration of solvent required to reduce the of hydroxylase activity to 50 %
K_M	Michaelis constant (M)
lauric acid	*n*-dodecanoic acid

Log P	logarithm of the partition coefficient of a given compound in the standard octanol-water two phase system. (Rekker, 1977).
Log P_{MEM}	logarithm of the partition coefficient of a given compound in coefficient in a membrane water two phase system
min	minutes
mol	Moles (6.23×10^{23} molecules)
$MgSO_4$	magnesium sulphate
n-alcohol	primary chain alcohol
P45011α	membrane associated enzyme that catalyses the biocatalysis of progesterone to hydroxyprogesterone
P450 BM3	cytochrome monooxygenase system in *B. megaterium* that catalyses the biotransformation of long chain fatty acids.
PDA	potato dextrose agar
PDB	potato dextrose broth
pH	$\log_{10}[H_30^+]$
p.s.i.	pressure, units of pounds per square inch
PMSF	phenylmethylsulfonyl fluoride
PP_i	inorganic phosphate
Progesterone	4-Pregen-3,20-dione
P. pyralis	*Photinus pyralis*
RO water	water purified by Reverse Osmosis
RPM	revolutions per minute
RMM	relative molecular mass
R. stolonifer	*Rhizopus stolonifer*
RLU	relative light units
sp.	spores
Sorbitol	D-glucitol
UV/vis	ultraviolet/visible
V_{MAX}	maximum rate of reaction (mol $s.^{-1}$).
wwt	wet weight
(w/v)	weight per unit volume

1. Introduction

1.1 Catalysis and Biocatalysis

1.1.1 Introduction to biocatalysis

A chemical reaction mediated by material derived from a biochemical process is termed biocatalysis. Biocatalytic processes are generally single step enzymatic reactions and so can be distinguished from fermentations, which involve primary metabolic pathways. The enzymes can be used in either a purified form, in a semi-pure form, for example as microsomal or peroxisome preparations, as crude extracts, or in whole cell systems. These protein based catalysts can originate from microbial, yeast, fungi, mammalian or plant cells. They can be used as free entities or an immobilised form.

1.1.2 Advantages of biocatalysis over chemical synthesis

The protein structures of enzymes with unique functional groups at the catalytic site. The protein structures of enzymes with unique functional groups at the catalytic site confer a high degree of specificity in the catalysis. On a molar basis enzymes usually have a faster turnover than the lower molecular weight catalysis used and organic reagents. The reactions usually take place under ambient conditions (at 20-40°C although some can withstand temperatures up to 100°C **(Ghogare and Kumar 1989).** Enzymes also require mild aqueous conditions rather than aggressive organic reagents, and they rarely need protection from a normal atmosphere of nitrogen and oxygen.

The major advantage of biocatalysis in terms of the fine chemical and pharmaceutical industry is that enzymes can exhibit high chemo-, regio- and enantioselectivity **(Halling, 1990).** One example where biocatalysis have replaced a chemical conversion is the production of 6-aminopenicillanic acid (6-APA). **Figure 1-1** shows the chemical and biochemical route **(Tramper, 1996).** A productivity of up to 2000 kg of 6-APA per kg of

immobilised enzyme is obtained with operating lifetimes in excess of 10^3 hr **(Tramper, 1996)**.

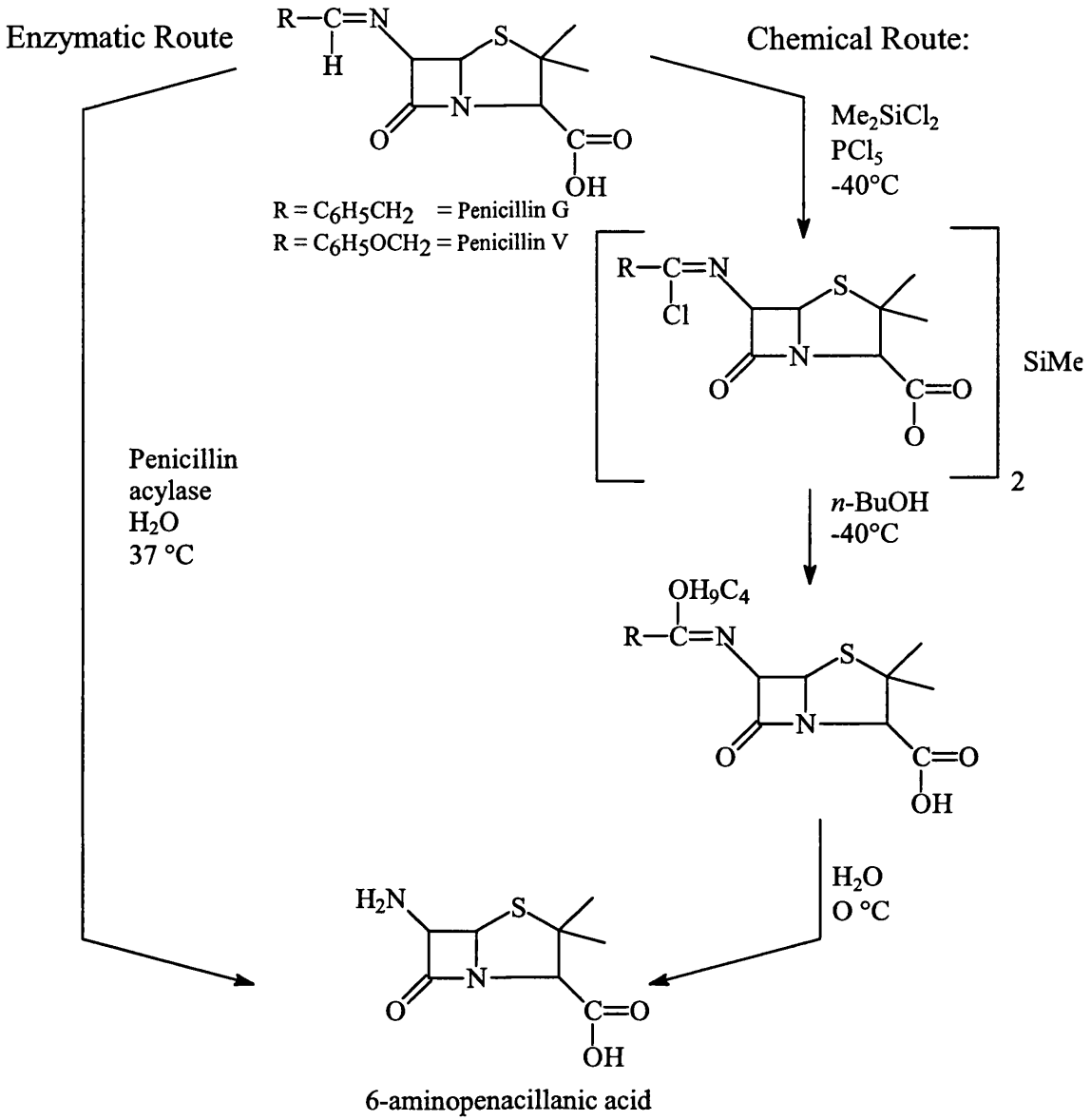


Figure 1-1 Production of 6-aminopenacillanic acid (6-APA) from penicillin. G or V by biocatalysis and chemical synthesis **(Savidge, T.A, 1984)**

1.1.3 Limitations of biocatalysts

Most enzymes are highly selective and will react with only one substrate. They have a low stability, are prone to thermal inactivation and show maximum activity in a narrow range of pH, temperature and ionic strength of media. If the reactant or product (in many cases

both) are hydrophobic or poorly water soluble, then the rate of the catalysed reaction and the concentration of products in the solution is limited. Hydrophobic organic solvents can be used to increase the solubility of reactants and products to enhance the reaction productivity **(Zaks and Klibanov, 1984).** However, these organic solvents tend to be toxic to enzymes **(Zaks and Klibanov, 1985).** Recent work is expanding the field of biocatalysis in water poor systems in anhydrous solvents, two-phase systems or in water miscible co-solvent systems **(Koskinen and Klibanov, 1996).**

1.1.4 Advantages of whole cell biotransformations

Some biochemical reactions require cofactors, which are readily available and are recycled in living cells. Oxidations and reductions often require complex nucleotides such a NAD^+ and $NADP^+$ or there reduced forms NADH and NADPH. ATP, which acts as an energy source is required to drive many cellular reactions **(Volt, *et al*, 1998).**

To maintain enzyme activity in the purified form, the cofactors must be recycled. This adds to the cost of the catalytic reaction and can be a limiting factor in the economics of the total process.

Another problem associated with purified biocatalyst is the difficulty in obtaining active preparations of membrane bound enzymes, for example, the cytochrome P450 hydroxylases. The enzymes interact with the biomembrane, which confers conformational information essential for catalytic activity. Thus, when the enzyme is removed from the hydrophobic support and the conformational information is lost, in many cases activity is reduced (for references see section 1.10). Generally, these enzymes are only stable when attached to a hydrophobic support **(Akita, 1996).** To maintain their activity after purification conformational state and the range of motions allowed must be maintained. This is very difficult to achieve and activity is usually lost when moving enzymes from biomembranes to an aqueous phase **(Akita, 1996).** One can imagine direct transport of enzymes from cellular membranes to synthetic hydrophobic supports but the engineering associated with the change is not well understood. Whole cells are also known to protect enzymes from contact with air-liquid and liquid-liquid interfaces preventing protein conformational changes and loss of activity **(Ushio, 1983).** In addition, the whole cell modulates protease expression, provides a controlled pH, and the ionic strength

environment important for enzyme activity **(Holland, 1998).** An example of an industrial whole-cell biocatalysis is the production of indigo **(Figure 1-2; Cameron & Tong, 1993).**

In the whole cell system there is no requirement for cofactor recycling. The high costs associated with purifying cytoplasmic enzymes, and the availability of active membrane bound enzyme systems, confer advantages of employing whole cell techniques.
For purified enzymes, the low solubility of hydrophobic substrates and products in the aqueous phase is a limitation of the whole cell biocatalytic process.

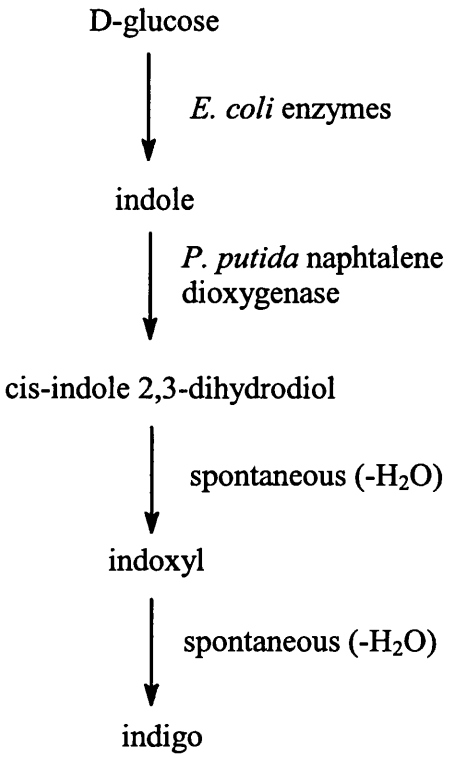


Figure 1-2 Biosynthesis of indigo by recombinant *E. coli* cells
(Endley, 1983)

The next section of this introduction (1.2-1.6) will consider aspects of the interaction of solvents with cell membranes in more detail, and this includes sections (1.5-1.6) on solvents as general anaesthetics, a process which is similar to the effects of solvents on biotransformations. This is followed by a section, (1.7) on the oxidative processes catalysed by the membrane bound enzymes of the cytochrome P450 group.
The effect of a series of alcohols on two of these important oxidative enzymes forms the experimental basis of this study, the rationale for which is set out in the final section (1.8) of this introduction.

1.2 Biocatalysts in non-aqueous solvents

The addition of water miscible organic solvents to an aqueous system can be used to increase the solubility of substrates and products of an enzymatic reaction **(Verger, 1997).** Thus, higher concentrations of both substrates and products can be contained. The rate of reaction increases and the equilibrium position of the reaction is shifted because products are more readily partitioned from the cell. The higher concentration of substrate improves the process economics. The disadvantage of using organic solvents lies in their toxicity to living cells. Life on earth is based on water. Organic solvents did not play a prominent role in the evolution of living systems. However, biological systems can produce large local quantities of water miscible organic acids and alcohols. The concentration of these hydrophilic compounds required for toxic effects are high. Very lipophilic compounds such as olive and vegetable oils are not toxic at any concentration to whole cells. Toxic solvents are typically more hydrophilic than long chain oils and fats and more hydrophobic than short chain alkanes and acids. Attempts to understand this toxicity initially centred on the solvents physical properties of the solvents **(Garbe, and Yukawa, 1991).**

1.2.1 Parameters reflecting solvent toxicity

Solvents can be described by physical characteristics such as: Molecular weight (M), shape, viscosity, surface tension and electrical properties such as specific conductivity, dielectric constant, dipole moment, the ability to form hydrogen bonds and polarisation.

Attempts have been made to correlate these physical properties with biocatalyst stability and solvent tolerance **(Maroni & Barbieri, 1989).**

1.2.1.1 The Hilderbrand solubility parameter

The Hilderbrand solubility parameter is defined as the square root of the cohesive density of a solvent **(Hilderbrandt *et al.*, 1970)**, and is an indication of the hydrophobicity of a solvent. **(Brink & Tramper 1985)** correlated the activity of calcium alginate immobilised *Mycobacterium sp.* to the Hilderbrand solubility parameter **(Figure 1-3)**. They found a higher correlation with the activity when the Hilderbrand solubility parameter was linked to molecular weight (M).

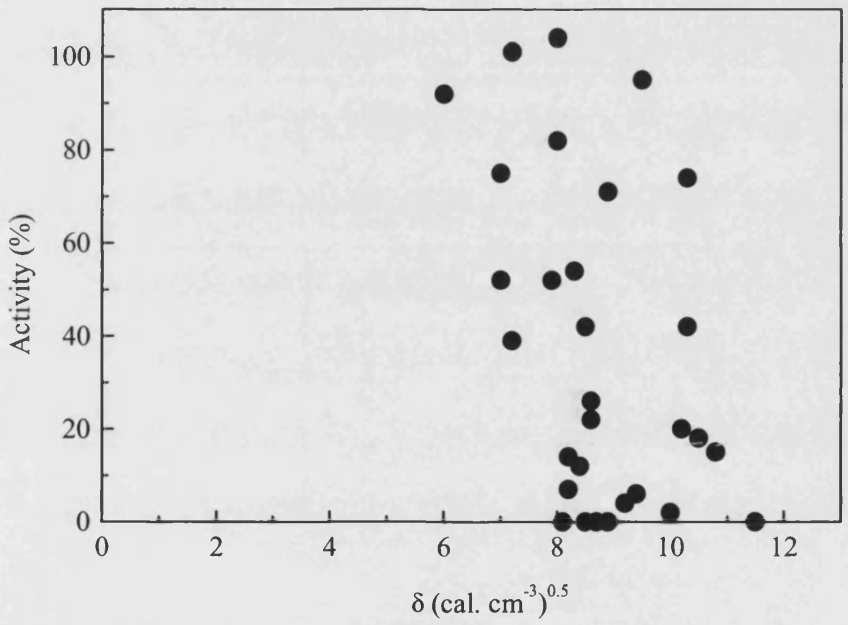


Figure 1-3 Activity retention of epoxidising, immobilised cells exposed to different organic solvents versus the Hilderbrandt solubility parameter (δ).

It was deduced that the high activity retention was usually favoured by a low solvent polarity in combination with a high molecular weight. The key parameter in the Hilderbrandt solubility expression **(Equation 1-1)** is the solvent heat of vaporisation (ΔH_v). ΔH_v strongly depends on polar interactions between solvent molecules. So, for

relatively apolar solvents versus the Hilderbrandt solubility parameter is relatively insensitive to differences in polarity.

$$\delta = \left(\frac{E}{v}\right)^{0.5} = \left(\frac{\Delta H_v - RT}{v}\right)^{0.5}$$

Equation 1-1 ΔH_v is the latent heat of solvation, (a measure of cohesive energy), v is the molar volume, R the gas constant, T is the absolute temperature (°K). (Laane *et al,* 1987).

1.2.1.2 Partition of Solvents into octanol (Log P)

Another more direct parameter reflecting the polarity of solvents is Log P, defined as the logarithm of the partition coefficient (P) of a given compound in the standard octanol water two-phase system **(Equation 1-2)**.

$$P = \left(\frac{[Solute]_{octanol}}{[Solute]_{water}}\right)$$

Equation 1-2 Partition coefficient of a given compound in the standard octanol water two-phase system

Log P values can be determined experimentally or calculated from hydrophobic fragmental constants **(Rekker, 1977). (Laane *et al.*, 1985)** re-plotted the data from **(Brink & Tramper 1985)** using calculated log P values. They obtained a higher correlation between the activity and the solvent polarity when log P values were used **(Figure 1-4). (Laane *et al.*, 1985)** also re-plotted the data from **(Playne & Smith 1983)** and found a clear correlation between log P and in that case the relative gas production activity of suspended anaerobic cells. The classic S shape of these log P activity plots suggested that the cells were strongly inactivated by solvents with log P values of between 2 and 6. This type of

curve was obtained with bacterial cells **(Harrop *et al.*, 1989)** and isolated enzymes **(Zaks and Klibanov, 1985)**.

In a bioreactor, the choice of solvent affects its compatibility with the microorganism. Elucidation of the mechanism of inhibition however cannot be obtained using the empirical data. An investigation of the molecular interaction of solvents with proteins and the interaction of membranes may provide information relating to the effect of solvents on the membrane bound enzymes, which is dependent on Log P. This is discussed further in section 1.3.

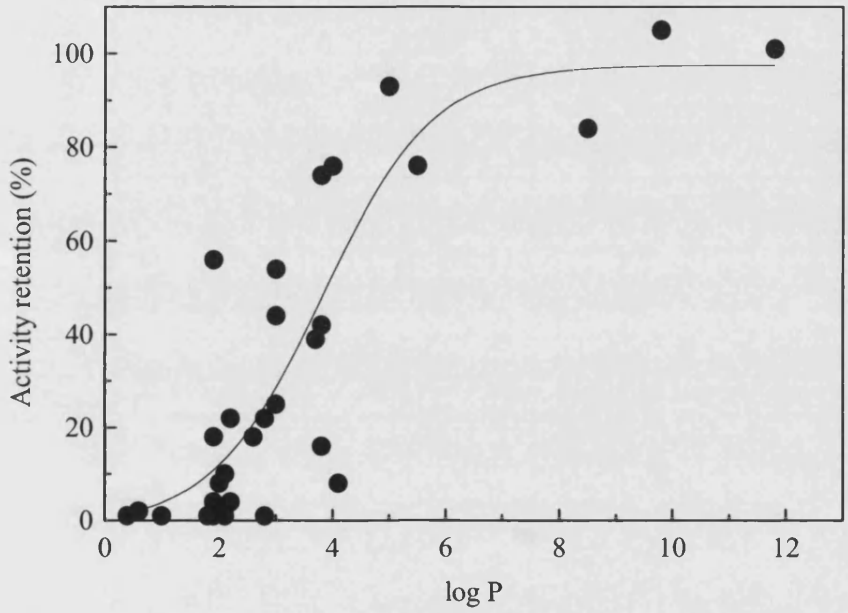


Figure 1-4 Activity retention of epoxidising, immobilised cells exposed to different organic solvents verses log P.

1.3 Effect of solvents on enzymes

In this section, the effect of organic solvents on purified enzymes is discussed. The four types of water poor systems are introduced and the advantages of removing water from the bulk phase of a two phase system are illustrated.

1.3.1 The importance of water for enzyme activity

Water is an absolute requirement for the catalytic activity of all enzymes, including those located within the cell membranes **(Klibanov, 1986)**. Water is important in all non-covalent interactions that help to maintain the conformation of enzymes, necessary for activity. It is easy to understand the importance of water close to the protein, but what function does the water in the bulk environment play and what effect does the solvent have on the water concentration? It is assumed that the function of water is limited to forming bonds with polar amino acids on the enzyme surface. If the bulk water is removed by an organic environment, the enzyme will not be affected **(Klibanov, 1986)** and the systems are called reduced water systems.

1.3.2 Reduced water systems

There are four main types of water poor systems; almost anhydrous solvents **(Klibanov, 1997)**, aqueous organic two phase mixtures **(Brink *et al.*, 1988)**, enzymes in reversed micelles **(Khmelnitsky *et al.*, 1992)** and enzymes contained within water-miscible organic co-solvents such as methanol and DMSO **(Klibanov, 1996)**. In the first three cases the enzymes are situated in an aqueous phase, which is surrounded by an organic phase. In the last case bulk phase water is removed and replaced by the organic solvent. The first three cases are immediately important in relation to the microorganisms. Anhydrous solvents have more relevance when purified enzymes are used. In this case a low concentration of water, $< 1\%$, remains and this seems to be important for enzyme function **(Klibanov, 1996)**.

1.3.3 Vital water

Solvents with log P > 5 tend to be non-toxic. This can be related to the ability of the water in the microenvironment of the enzyme to partition into the bulk phase. Highly hydrophobic solvents have a low solubility for water and so the tendency for the 'essential water' to be striped away from the enzyme is low and the active conformation is maintained **(Klibanov, 1986)**. Solvents with lower log P values have a higher solubility for water and can remove the vital water more readily. But very low log P solvents can be used as co-solvents **(Bell, *et al,* 1995)**.

1.3.4 Advantages of removing bulk water

Most enzymes have an excess of hydrophilic groups on the surface, and tend to have a low solubility in an organic phase. This low solubility leads to the 'pH memory effect.'. All enzymatic reactions in aqueous environments are highly pH sensitive. Enzymes in organic solutions remember the pH of the last aqueous media. At a molecular level the ionisation state of the ionogenic groups on the surface of the enzyme are conserved and the activity associated with that pH is also conserved.

Another effect of reducing the volume of water available to the enzyme is the increase in the conformational rigidity. For example, porcine pancreatic lipase does not react with large substrates in anhydrous organic solvents. In this example solvent restricts conformational movement that allows the active site to relax in order to accommodate new substrates **(Klibanov 1986)**.

In water, enzymes are irreversibly inactivated at high temperatures **(Ahern & Klibanov, 1985)**. Over a small rise in temperature, the amplitudes of all bond motions increase and the protein will reversibly unfold. Any loss of activity associated with this rise in temperature will be restored as the temperature is reduced.

Larger increases in temperature will result in formations of other functionally incorrect structures. Destruction of disulphide (S=S) bonds via β-elimination, deamination of asparagine and glutamine residues and hydrolysis of peptide bonds at aspartic acid residues are all possible chemical changes, but they all require water. If bulk water is removed the inactivation of enzymes at high temperature should be reduced in organic solvents. This can be seen with porcine pancreatic lipase at 100 °C **(Zaks & Klibanov, 1984)**.

1.4 Effect of solvents on living cells

The effects of organic solvents on whole cell biocatalysts cannot be discussed without an overview of the structure of whole cells. This section describes the mechanics of the bacterial cell wall and the differences between gram negative and gram positive bacteria. Membrane proteins and the Fluid Mosaic Model of the membrane are also reviewed. The section continues with the interactions of solvents with the membranes, including a further development of log P in relation to the solubility of solvents in the biological membranes. The adaptive mechanisms and evolution of microorganisms to natural and xenobiotic solvents ends the section.

1.4.1 Architecture of whole cells

Eukaryotic cells are highly developed and have a nucleus containing DNA surrounded by a nuclear membrane **(Volt *et al,* 1998).** The cytoplasm surrounds the nucleus and is subdivided into a variety of compartments. In addition, biological membranes surround mitochondria. The endoplasmic recticulum, ribosomes and golgi apparatus are all organelles found in animal cells and are important in enzyme production and modification. A semi-permeable phospholipid membrane surrounds the cell.

Prokaryotic cells are less developed. They have no mitochondria and the DNA is not contained within a nucleus. Bacterial membranes consist of several layers. Two layers are osmophilic, each about 2-3 nm thick, surrounded by a layer 4-5 nm thick. The membranes of bacteria, plants, and animals show considerable resemblance in their general structure.

The bacterial membrane is rich in phospholipid. Membrane lipids constitute about 70-90 % of the cellular lipids, whereas the membrane constitutes only 8-15 % of the cellular dry weight. The cytoplasmic membrane consists of a lipid bilayer, with the hydrophobic groups exposed on the exterior surfaces. The bilayer has proteins incorporated into it. These are the integral membrane proteins, which can be considered as floating in the membrane matrix, some completely traversing it, whilst others are partially immersed. Other proteins are attached to the membrane and are described and peripheral membrane proteins. Some membranes are covered on one or both surfaces by a network of elongated proteins **(Schlegel, 1988).**

The membrane should be considered as a very soft, deformable, almost fluid structure. Isolated membranes have a tendency to round up into vesicles with fragments fusing along their edges **(Schlegel, 1988)**.

1.4.2 Bacterial cell walls

Microbial cell envelopes are elastic, highly complex structures. The cell envelope is composed of the cell wall and cytoplasmic membrane. The chemical structure of the cell wall varies between genera but the cytoplasmic membrane remains essentially the same. The cell envelope protects the microorganism against osmotic shock, allowing them to live in hypotonic (less than intracellular salt concentrations) environments that would otherwise cause them to swell osmotically until their plasma (cell) membranes lysed **(Volt *et al*, 1998).**

1.4.2.1 Gram-positive and gram negative bacteria

Bacteria can be classified as gram positive or gram negative depending on whether or not they take up gram stain. Gram-positive bacteria have a thick (~250 Å) cell wall surrounding their plasma membrane, whereas gram-negative bacteria have a thin (~30 Å) cell wall covered by a complex outer membrane **(Volt *et al*, 1998).**

Gram negative microorganisms also have a periplasmic space, which separates the cell wall from the plasma membrane. Lipopolysaccharides (LPS) are randomly positioned and cover 30-40% of the outer surface on the outer membrane. They are highly hydrophilic and so provide protection against the passage of hydrophobic molecules **(Bull *et al.*, 1982).** The cell wall is composed of a highly cross-linked peptidoglycan structure. It can vary between 0.8 and 30 nm in diameter and forms an anionic sieve-like structure enabling lipoproteins to have a rigid base to anchor on. Solvent passage through the peptidoglycan layer occurs unhindered. When a solvent interacts with the cytoplasmic membrane, expansion may occur and microscopic observation has showed membrane expansion **(Bull, 1982).**

1.4.2.2 Lipids and membranes

The function of lipids is to organise biological processes by compartmentalising them. They are substances of biological origin that are soluble in organic solvents such as

chloroform and methanol but are only sparingly soluble, if at all, in water. Lipids can aggregate to form micelles and bilayers. Bilayers are the structural basis for biological membranes **(Binder, *et al.*, 2004).**

In aqueous solutions, amphiphilic molecules such as soaps and detergents form micelles. This molecular arrangement eliminates unfavourable contacts between water and the hydrophobic tails of the amphiphiles **(Binder, *et al.*, 2004).**

1.4.3 Biological membranes

Biological membranes are composed of proteins associated with a lipid bilayer matrix. Their lipid fractions consist of complex mixtures that vary according to the membrane source and to some extent with the diet and environment of the organism **(Eichler and Irihimovitch, 2003).**

1.4.4 Membrane proteins

Membrane proteins can be classified according to how tightly they are associated with the membranes.

Integral or intrinsic proteins are tightly bound to membranes by hydrophobic interactions and can be separated from them by treatment with agents that disrupt membranes. These include organic solvents, detergents, and chaotropic agents (ions that disrupt water structure). Cytochrome $P450_{11\alpha}$ in *R. stolonifer* is an example of an integral membrane protein. **(Domanski & Halpert, 2001).**

Peripheral or extrinsic proteins can be dissociated from membranes by relatively mild procedures that leave the membrane intact, such as exposure to high ionic strength salt solutions, or pH change. Cytochrome *c* is an example of a peripheral protein that is stable in aqueous solution. **(Kim *et al.*, 2000).**

1.4.5 Fluid Mosaic Model of Membrane Structure

In this, the Fluid Mossaic Model of Membrane Structure **(Slinger, 1974)**, integral proteins are able to freely move laterally over a two-dimensional 'sea' of lipid unless associations with other cell components restrict their movements. This model has been verified experimentally by the fusion of cultured mouse cells with human cells by treatment with

Sendia virus to yield a hybrid known as a hetrokaryon. After 40 min. at 37 °C cell proteins of the mouse, cell and human cell were observed to be randomly distributed over the hetrokaryon **(Slinger, 1974)**.

1.4.6 Partitioning of solvents into biological membranes

The measure of log P can be used to estimate values for partitioning of solvents in to membrane-buffer systems **(Osborne *et al.*, 1992)**. Hydrophobicity however is not the only parameter that affects solvent solubility into membranes. The molecular structure of a solvent will alter its ability to migrate into a phospholipid bilayer. Amphiphilic molecules that have both hydrophobic and hydrophilic centres have structures that mimic the phospholipid nature of cell membranes and can dissolve more readily **(Saunders, 2003).** An example being the phenols, which are relatively more toxic than other compounds **(Kitagawa *et al.*, 1990)**. The composition of the membranes also influences the solubility of the compound into the membrane. For example, the partition coefficient of lindane is 50 times higher in liposomes of dimyristoyl-phospatidylcholine (C14:0) than in liposomes of diasterroylphosphatidyl-choline (C18:0) **(Antunes-Madeira & Madeira, 1989)**.

1.4.7 Influence solvents on lipids and membranes

Once a solvent molecule has partitioned in to the membrane it will be incorporated into the structure and hence disturb the integrity and the complex system of phospholipids and proteins. Techniques for investigating membrane integrity include the use of fluorescence labelled liposomes. Any expansion of the membrane caused by an influx of solvent will tend to dilute the radio labelled probe and indicate membrane swelling as a function of the reduction in radio signal **(Saunders, 2003).**

1.4.8 Adaptation to natural solvents

The resistance of microorganisms to ethanol mediated by changes in the structure has been reviewed **(Ingram, 1984)**. The composition of the membranes is altered either by increasing the chain length of the fatty acid chain, or by increasing the proportion of *Cis*-monounsaturated fatty acids. Both mechanisms have been encountered in *Lactobacillus homohiochii* and *L. heterohiochii* **(Ingram, 1984)**.

The ratio of unsaturated to saturated fatty acids is increased in the presence of ethanol in *E. coli* and liposomes with a high content of unsaturated fatty acids were shown to exhibit increased fluidity. Membranes from cells grown on ethanol have a higher proportion of membrane proteins **(Heipieper, Keweloh & Rehm, 1991).** The increase in the protein lipid membrane has been shown to increase the rigidity of the cellular membrane.

1.4.9 Adaptation to xenobiotic solvents

Increasing the degree of saturation of membrane lipids is one method of adaptation to xenobiotic compounds and has been shown for the phenol-degrading strain *Pseudomonas putida* S12 **(Isken S, Derks A, Wolffs PFG, *et al.*, 1999).**

Cis- to trans- isomerisation of fatty acids is a method of adapting cell membranes to toxic solvents that do not occur in the natural environment. In the presence of phenols *P. putida* P8 was found to adapt by altering the proportion of cis and trans isomers of fatty acids **(Loffhagen N, Hartig C, & Babel W, 2001).** It remains to be seen if the partition coefficient of the solvents is strongly affected by the composition of the membrane.

Solvent tolerant organisms could be used in the field of biocatalysis. The use of organisms with complete tolerance to solvents with a low solubility in water such as toluene, styrene, tetralin and terpenes will promote the application of whole cells in such systems. This will reduce the cost of biocatalysis that require cofactors such as NAD and ATP **(Section 2).**

1.5 Solvents as anaesthetics

An examination of the effect of solvents on whole cells has shown that the Log P value is useful in determining toxicity. This can be related to the solubility in biological membranes, but for some molecules this correlation breaks down **(Antunes-Madeira & Madeira, 1989)**. An understanding of the interaction of organic solvents with biological membranes is important in the field of anaesthesia where organic solvents can be described as anaesthetic like molecules. This section introduces the lipid theory of anaesthesia. The problems with this theory and the possible solutions are discussed below.

1.5.1 Meyer and Overton

In the 1890s Hans Meyer and Ernst Overton showed that the potency of anaesthetics increased in proportion to its olive oil water partition coefficient **(Equation 1-3), (Seeman, 1972)**

$$P = \left(\frac{[Solute]_{olive\ oil}}{[Solute]_{water}} \right) \propto \text{anesthetic potency}$$

Equation 1-3 Partition coefficient P, of a given compound in the standard octanol water two-phase system.

A better correlation of anaesthetic potency with solubility was achieved with an octanol water system **(Franks & Lieb, 1982)**. A double log plot of potency in the aqueous phase and the octanol water partition coefficient yields a straight line with unit slope **(Franks & Lieb, 1982)**. The anaesthetic site has similar characteristics to octanol and anaesthetic potency can be predicted from Log P. Although the site is amphiphilic, having both hydrophilic and hydrophobic properties, and the interaction is less affected by the size, shape or chemical characteristics of the solvent.

1.5.2 What is the target for anaesthetics?

Since Meyer and Overton discovered a correlation between the anaesthetic potency of simple molecules and partitioning into fat-like solvents, the traditional view has been that anaesthetics act by disrupting the bulk structure of the lipid portions of nerve membranes **(Janoff, Pringle & Miller, 1981)**.

The lipid properties that are modified differ from theory to theory and include volume expansion **(Richards, 1980)**, increasing membrane fluidity **(Franks & Lieb, 1982)**, increasing membrane thickness **(Haydon *et al.*, 1984)**, increasing membrane surface tension **(Miller, 1985)**, and creating polar dislocations in lipid bilayers **(Bangham and Hill, 1986)**. The effect of anaesthetics on these membranes has been shown to be very small and can be reproduced by a small (1 °C) change in temperature. This evidence lead to a new hypothesis that it was the boundary lipids surrounding membrane proteins that are preferentially affected **(Franks & Lieb, 1984)**. However recent work suggests that this is not correct **(Abaji *et al.*, 1993)**.

Most anaethetic drugs whose mechanisms have been established are know to act by binding directly to proteins. Evidence that general anaesthetics might also act in this way came with the demonstration that the activity of a soluble lipid-free enzyme, firefly luciferase, could be inhibited by a wide range of anaesthetics at IC_{50} concentrations very close to animal EC_{50} values. These observations are not only evidence for the protein binding mechanism of anaesthesia, but provide a simple explanation to the Meyer-Overton correlation in the form of anaesthetic binding sites **(Franks & Lieb, 1986)**.

1.5.2.1 Molecular cut off effect

Although anaesthetic potency increases with Log P, there is a size limitation. This is demonstrated by the so-called cut-off effect **(Franks & Lieb, 1986)**. As a homologous series of anaesthetics is ascended, aqueous phase potencies increase until they become totally ineffective anaesthetics **(Pringle *et al.*, 1981)**. One explanation for this finding is that the site has a finite size and simply excludes large molecules. This can account for the inactivity of dodecanol relative to tetradecanol. The cut off effect can be most simply explained by anaesthetics binding to protein pockets or clefts with fixed dimensions **(Franks & Lieb, 1993)**. Explanations in terms of lipid theory have been more difficult to accommodate. It was suggested **(Seeman, 1972)** that the solubility of long-chain alcohols

was simply not sufficient in bilayers, but this has been shown to be an incorrect assertion by experiment **(Franks & Lieb, 1991)**.

Evidence that the mechanism of anaesthesia is via direct protein interactions comes from studies with chiral anaesthetics. These compounds show no stereospecific effect on bulk lipid bilayers but are stereospecific with respect to their anaesthetic abilities **(Franks & Lieb, 1991)**.

The current evidence **(Franks & Lieb, 1991)** indicates that general anaesthetics act by binding directly to proteins.

It is also suspected that the binding sites of these proteins are hydrophobic pockets exposed to water rather than interfacial sites exposed to lipid hydrocarbon chains. Water filled pockets might be expected to bind hydrophobic anaesthetics more tightly. It is generally agreed that the ultimate target of anaesthetics is a membrane protein even if the interaction is mediated by the lipid bilayer. In the next section, the different possible protein targets are discussed effect **(Franks & Lieb, 1987)**.

1.6 Protein target of anaesthesia

It is known that anaesthetics act by selectively targeting synaptic ion channels or the systems that regulate them **(Miller, 2002).** Their action at the molecular level however remains uncertain. There are two sites that have been promoted as a possible receptor for anaesthetic molecules: proteins and lipids **(Sureda & Mallol, 2003).**

The effects of anaesthesia on the central nervous system (CNS) have been explored over the last decade and these studies have focused largely on nerve membranes, and in particular on neuronal ion channels and the systems that regulates them. Current evidence suggests that the anaesthetic effect originates from an alteration in synaptic or axonal transmission. A synaptic target could be either pre or post-synaptic and the effect of anaesthetics on synaptic sites could be to stimulate inhibitory synapses or inhibit excitatory synapses. An important question that remains unanswered is the molecular effects of anaesthetics, whether they perturb membrane lipids or as evidence that is more recent indicates, bind directly to membrane proteins **(Sureda & Mallol, 2003).**

1.6.1 The effect of anaethetics on Voltage-gated ion channels

Voltage-gated ion channels are regulated by changes in membrane potential and include Na^+, K^+ and Ca^{2+} channels.

1.6.1.1 The effect of anaethetics on Na^+ and K^+ channels

The interactions of many simple organic compounds with the Na^+ and K^+ channels involved in the generation of an action potential have been described for the squid giant axon **(Elliot & Haydon, 1989).** At physiologically relevant concentrations however, both the Na^+ and K^+ channels are insensitive to solvents and axonal conduction is unaffected at anaesthetic concentrations.

Voltage-gated Na^+ or K^+ channels, appear unlikely candidates for major anaesthetic targets **(Elliot & Haydon, 1989).**

1.6.1.2 Ca^{2+} channels

Voltage-gated Ca^{2+} channels (T, L, N and P) share sequence homology with voltage gated Na^{+} and K^{+} ion channels and are members of the same super-family. N and P type channels are found mainly in neurones and these appear to be most directly involved in the pre-synaptic release of neurotransmitters from control nerve terminals **(Takahashi & Momiyama, 1993)**. The effects of volatile anaesthetics have been extensively investigated and most studies show that these channels are extremely insensitive to these agents **(Franks & Lieb, 1993)**. Barbiturates have been shown to inhibit Ca^{2+} channels, but usually with half-maximal inhibitory concentrations (IC_{50}) more than three fold in excess of the free aqueous concentrations that cause general anaesthesia **(Nishi & Oyama, 1983)**. In summary, voltage gated Ca^{2+} channels appear to be generally insensitive to clinically used anaesthetics.

1.6.2 Ligand-gated ion channels

Ligand-gated ion channels respond to binding of a specific molecule and include glutamate, nicotinic acetylcholine, and $GABA_A$ receptors **(Harrison, *et al.*, 1997).**

L-Glutamate is probably the major excitatory neurotransmitter in the vertebrate CNS. There have been few modern studies with clinical general anaesthetics on its ionotropic receptor channels (iGluRs), which mediate fast excitatory transmission. They are classified by their selective agonist NOMAD (*N*-methyl-D-aspartate), KA (Kainate), and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionoic acid). Ketamine has been shown to exert its anaesthetic effect by inhibiting the NOMAD receptor. The NOMAD receptor contains at least five distinct sites and is opened when occupied by an agonist, such as NOMAD. When open it allows an influx of Ca^{2+} and Na^{+} into the cell. In contrast, to ketamine all volatile agents are ineffective at modifying the iGluRs to give any anaesthetic effect **(Wakamori *et al.*, 1991)**.

The nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction is the most thoroughly studied of all neurotransmitter receptors. It has been shown that the simplest anaesthetics at high concentrations can stabilise a desensitised form of the receptor. At relevant doses however other effects are observed that vary between agents. Among the volatile anaesthetics the fluorinated ethers isoflurane and enflurane are particularly

effective at inhibiting the receptor, with the primary effect at the single-channel level being to reduce the mean open time **(Yushmanov, *et al.*2003).**

Intravenous agents, barbiturates, and short chain alcohol's have been shown to; increase the decay rate of miniature endplate currents, reduce single channel mean open time in denervated rat skeletal muscle **(Dilger *et al.*, 1992)**, and inhibit nerve response to stimulus respectively.

Although nAChRs are sensitive to a number of different anaesthetics, it is not clear yet that how their inhibition contributes to general anaesthesia?

General anaesthetics may act by potentiating inhibitory synaptic transmission, and the $GABA_A$ receptor channel has been considered a potential target. GABA (γ-aminobutyric acid) is the most important inhibitory neurotransmitter in the brain and most anaesthetics are very effective at potentiating responses to GABA. The major action of pentobarbital and most other anaesthetics is to shift the GABA response curve to lower concentrations, so peak Cl^- currents elicited by high levels of GABA are virtually unaffected. Anaesthetics tend to enhance and hence prolong receptor binding of GABA released pre-synaptically from vesicles in brief but high concentration pulses.

Single channel studies with volatile agents **(Yeh *et al.*, 1991);** barbiturates and **propofol (Hales & Lambert, 1991)** show no changes in channel conductance, but the channel open time increases, consistent with the prolongation of post-synaptic currents. Thus the molecular basis for the potentiation of GABA action by anaesthetics is an increased probability for the $GABA_A$ receptor channel to be in the open state. The extent to which this is due to enhanced GABA binding, or to changes in channel gating, remains to be determined and may depend on the anaesthetic.

Generally almost all agents at their half-maximal effective concentrations EC_{50} enhance the current induced by low levels of GABA by over 50 %. Although uncertainties remain as to the extent to which intact GABA synapses are functionally potentiated, there is now an impressive body of evidence that points to the $GABA_A$ receptor channel complex as a major target for most general anaesthetics. If this is a direct effect on the ion channel or an indirect effect through an action on the membrane is yet to be determined.

1.6.3 Other inhibitory ion channels

Whereas GABA is the most important neurotransmitter in the brain, glycine plays a major role in the spinal cord and lower brainstem. However, no comprehensive anaesthetic studies have been carried out on the glycine receptors.

Inhibitory synapses are also candidates for explaining the well-known pressure reversal of general anaesthesia. For example, the effects of pressure resemble those of strychnine **(Boser-Riley, Daniels & Smith, 1988)**, a glycine antagonist. As pressure almost invariably depresses synaptic transmission *in vitro*, pressure reversal could, in principle, be explained by pressure blocking inhibitory synapses, but there is now less conviction that pressure reversal will provide fundamental clues as to how anaesthetics act. The phenomenon of pressure reversal may prove to be incidental to the mechanisms of general anaesthesia.

1.6.4 Synaptic target

Although it is clear that anaesthetics can have substantial affects on post-synaptic membranes, the extent to which they act pre-synaptically is difficult to assess. Studies **(Takenoshita & Takahashi, 1987)** suggest that at relevant concentrations of halothane and thiopental neurotransmitter release might be inhibited by about 20 %. As a whole, it is likely that some anaesthetic inhibition of transmitter release occurs at many synapses, although the effects are small.

1.6.5 Second messenger systems

Second messengers regulate the activity of many enzymes and ion channels, sometimes by direct binding but more often by phosphorylation. Free intercellular Ca^{2+} is an important second messenger and it has been thought that anaesthetics might disrupt neuronal function by increasing its concentration. It has been shown however, that there is no significant sustained effect on resting intracellular Ca^{2+} at anaesthetic concentrations **(Daniell & Harris, 1988)**. The inhibitory effects have also been ascribed to blocking of voltage-gated or receptor-operated Ca^{2+} channels, inhibition of inositol triphosphate ($InsP_3$) production or depletion of internal Ca^{2+} stores.

Another branch of the phosphadyl inositol second messenger pathway produces diacylglycerol, which activates protein kinase C (PKC). The effects of anaesthetics on diacylglycerol production are not known to date, but its PKC target can be inhibited by anaesthetics. Liver cytochrome P450-mediated metabolism of arachidonic acid, an eicosanoid second messenger that can also be derived from inositol phospholipids, is inhibited very selectively by a wide range of anaesthetics. There is little hard evidence that second messenger systems are involved in general anaesthesia. At high enough concentrations anaesthetics can substantially affect many second messenger systems but the few studies made at relevant concentrations suggest that they may be relatively insensitive to anaesthetic perturbation.

1.7 Cytochrome P450

Cytochrome P450 superfamily is widely distributed in animals and plants. There is perhaps no better example of the exploitation of the heme cofactor for such an abundant variety of chemical reactions than the P450 cytochromes (P450s). These enzymes use the same basic construction and chemistry to catalyse a wide variety of reactions of an enormous library of chemicals. Among the many types of chemical reactions catalysed by the P450s are hydroxylation, epoxidation, dehydrogenation, sulphoxidation, dehalogenation and N-, S- and O-dealkylation. P450s can also catalyse reduction of e.g. alkyl halides, and nitric oxide, isomerisation of prostaglandin H2 **(Hecker *et al.*, 1989)**, and dehydration **(Song & Brash, 1991)**.

Many aspects of this research on these enzymes have strong medical implications. For instance, many prescribed drugs are metabolised by the hepatic P450 systems and may also act as inducers of the production of the relevant isoforms (usually at the gene level). Barbiturates are a classic example of the P450 induction phenomenon **(Alterman *et al.*, 1995)**. Analysis of the P450s and their substrate specificity profile is central to the comprehension of the efficacy and safety of numerous drugs. The basic roles of the P450s in human metabolism are still being deciphered and the means by which the P450s are regulated (at both the gene and protein level) is of major importance. A very large number of human P450s are involved in steroid metabolism and are subject to regulation by hormones. An example of which is the adrenal gland mitochondrial P450scc which catalyses the oxidative cleavage of the cholesterol side chain to produce pregnenolone. This is the initial step in the mammalian steroid synthesis pathway **(Hanukolglu, *et al.*, 1993)**.

Since the discovery of the first P450s there has been a large increase in the number of P450s identified. Multiple membrane-bound forms have been identified from eukaryotic sources and a wide range of important biological functions are attributed to them including human liver isoforms, the oxidation of numerous drugs (including ibuprofen, codeine and caffeine), the interconversion and metabolism of steroids **(Park, *et al.*, 1995)**. An increase in the exposure of mammals to drugs and other environmental xenobiotics occurred over the last 50 years, and the P450s played an important protection role against these

compounds. The multiple forms of mammalian P450s have a wide and often overlapping substrate specificity profile. The oxidation of the xenobiotic materials catalysed by P450s generally results in an increase in the hydrophilicity of these compounds. The reaction also facilitates their excretion into the aqueous solutions of urine and/or bile. The P450s can transform otherwise inert chemicals into highly reactive and possibly mutagenic/carcinogenic derivatives, e.g. the biotransformation of benzo-a-pyrene, a relatively inert component of cigarette smoke, into a carcinogenic epoxide derivative **(Hadley et al., 1982)**. The arrival of gene sequencing techniques 20 years ago led to the accumulation and comparison of P450 amino acid sequences. In recent years, improved techniques for gene isolation and characterisation as well as genome sequencing projects have provided a comprehensive databank of P450 sequences for analysis. The P450s have been organised into an enzyme superfamily with divisions into families based on protein sequence homology. Within the superfamily, members of the same family generally share >40 % identity with one another and members of the same sub-family show even stronger identity. The number of sequences documented now exceeds 500 **(Nelson *et al.*, 1996)**. Clearly, a great deal of protein chemical, structural and enzymological characterisation remains to be performed with the majority of the P450 enzymes. The vast numbers of P450s in certain species is an indicator of the versatility of their chemistry and of their importance to the survival of higher organisms. Analysis of P450 evolution through amino acid sequence alignments suggests that fatty acid metabolising forms were among the earliest to develop. The fact that P450 has been discovered in archaebacteria, ancient organisms, indicates that P450s may have served useful roles in very early life forms.

1.7.1 Oxygen activation

Most of the biochemical transformations catalysed by the P450s result from an initial substrate monoxygenase reaction and the chemistry of oxygen activation catalysed by the P450s has always been a topic of great intrigue. The process can be described using P450cam as the model system. The heme iron of P450 is ligated by a cysteine as the fifth ligand. The sixth position is left vacant for ligation of oxygen during catalysis, but may be occupied by a water molecule in the resting state. Substrate binding to the P450 provides the starting point for the monooxygenase cycle. The heme iron is ferric and low-spin (S = 1/2) until binding of camphor. Thereafter, active site water is displaced by the substrate

and the iron becomes high-spin (S = 5/2). Associated with the spin-state shift is an increase in the redox potential of the heme (from approx. 300 mV to -170 mV). The redox potential shift permits one electron reduction by reduced putidaredoxin **(Sligar, 1974)**. Oxygen then binds to the ferrous heme iron, forming a ferrous-superoxide species referred to as "oxyP450" **(Sligar, 1974)**. The delivery of a second electron to the heme (also from putidaredoxin) precipitates an extremely rapid substrate oxygenation process, the mechanism of which is still the subject of some controversy. A likely mechanism involves reduction to a ferric-peroxy species, which may also exist in a ferrous-superoxide form. The subsequent delivery of two protons to the reaction centre results in the loss of one atom of oxygen in the formation of a water molecule and the production of a transient high-valent ironoxo species. This is often presented as $[FeO]^{3+}$, but may be stabilised through a number of resonance structures. The abstraction of a hydrogen from the camphor results in formation of a substrate radical that collapses via an oxygen rebound to generate a ferric enzyme-product complex. Dissociation of the 5-OH camphor returns the P450 to its resting state. The ability of the P450s to catalyse the oxidation of both NAD(P)H and substrate explains their older title of mixed function oxidases. While P450cam remains the most intensively studied of all the P450s, there has also been intensive research on the P450 BM3 system over the last decade. The P450 BM3 system may provide an experimentally tractable model for the entire class II P450 electron transfer chain (diflavin P450 reductase and P450) in a single polypeptide. At 119,000 Da, the fatty acid hydroxylase P450 BM3 is the largest P450. It also has the highest catalytic centre monooxygenase activity of any P450 - with rates of up to 4600 min^{-1} reported for the oxidation of favoured substrates. While P450 BM3 will hydroxylate fatty acid of a variety of chain lengths, optimal activity is towards C15 and C16 molecules. Oxidation occurs near the terminal of the molecules. The ω-2 position is usually favoured, but oxidations can also occur at ω-1 and ω-3 **(Miura & Fulco, 1986)**.

1.7.2 Role in detoxification

Many eukaryotes and bacteria respond to the presence of a P450 substrate by inducing the production of the P450 responsible for its catabolism at the gene level. These response mechanisms provide organisms with the ability to cope with novel exposure to an array of xenobiotics and to changes in the concentrations of endogenous biochemicals. The

mammalian microsomal P450s are a first line defence mechanism for higher organisms, playing a vital role in the conversion of xenobiotic toxins. Many microorganisms have taken this a step further, utilising the P450s in metabolic pathways for the assimilation of energy contained within unusual carbon sources **(Sanglard & Loper, 1989)**.

P450s are b-type cytochromes, which derive their title from the absorbance peak associated with their carbon monoxide-bound ferrous forms **(Poulos *et al.*, 1995)**. The unusual absorbance properties derive from an iron-sulphur bond (through a conserved cysteine residue) which anchors heme into the active sites of the enzyme. While there is much variation in the amino acid sequences available for the P450s, the known structures exhibit significant similarity - all are a-helix rich constructions resembling triagonal prisms. The P450s have the ability to bind and activate molecular oxygen, breaking the dioxygen bond and catalysing the insertion of a single atom of oxygen into a wide range of organic molecules, often with high stereo- and regio-specificity. A vast number of P450s have been characterised at the DNA level, with partial and total nucleotide sequences (and, hence, translated amino acid sequences) for well over 300 forms identified by 1993 **(Nelson *et al.*, 1993)**.

1.7.3 Origins of cytochrome P450

The ancestral gene existed more than 3.5 billion years ago, predating drugs and animal-plant interactions by at least 1 billion years. An early role for oxidative enzymes in prokaryotes most likely involved energy substrate utilisation: insertion of oxygen into various inaccessible forms of carbon and other food sources, thereby rendering them accessible to further metabolism. It has also been proposed that the P450 enzymes, as well as other 'drug metabolising' enzymes, play an important role in maintaining the steady state levels of endogenous ligands involved in ligand modulated transcription of genes effecting homeostasis, growth, differention, and neuro-endocrine functions **(Nebert, 1991)**.

1.7.4 Characterisation

Due to the inherent difficulties in purifying and working with membrane-bound enzymes, fewer have been characterised at the enzyme level. However, the bacterial forms of P450 are all soluble enzymes and are more amenable to purification and characterisation by

protein, chemical and spectroscopic techniques. Most of our understanding of P450 structure and function has been deduced through studies of the bacterial forms.

Four P450 atomic structures have been solved to date, all of which are from bacterial sources. The first structure, the camphor hydroxylase P450cam from *Pseudomonas* was reported in 1987 **(Pooulos, Finzel & Howard)**. In 1993, the structure for the fatty acid hydroxylase P450 BM3 from *Bacillus megaterium* was reported **(Ravichandran *et al.*, 1993).** The structures of P450(terp), **(Hasemann *et al.*, 1994)** and P450eryf **(Cuppvickery & Poulos, 1995)** have also been solved.

1.7.5 Classification by redox partner

The cytochromes P450 can be divided into two classes, depending on the nature of their redox partners, which provide them with the two successive one-electron transfers required for their catalytic process.

1.7.5.1 Class I

Class I enzymes are the terminal oxidases of a 3-component electron transfer chain and are typified by the mammalian adrenal mitochondrial steroid hydroxylases and all but one bacterial form. The P450 is reduced by a small iron sulphur protein (ferredoxin) which, in turn, is reduced by an NAD(P)H-dependent FAD-containing ferredoxin reductase **(Munro & Lindsay, 1996).**

1.7.5.2 Class 11

The mammalian hepatic drug-metabolising forms typify class II P450s. In these cases a single FAD and FMN containing enzyme (an NADPH-cytochrome P450 reductase) catalyses electron transfers to the P450 **(Harikrishna, 1993).** The cytochrome P450 BM3 from *B. megaterium* ATCC 14581, is the only example of a bacterial class II P450. This cytochrome P450 is interesting since it is reduced by a eukaryotic-like P450 reductase that is fused to the P450 in a single polypeptide **(Narhi & Fulco, 1987).** In both classes of cytochromes the order of electron flow is NADPH -> FAD -> FMN -> heme.

1.7.6 Classification by solubility

Another method of classifying the P450s is based on the solubility. All the prokaryotic members of the P450 superfamily are soluble and can be maintained in aqueous solution at high concentrations. Eukaryotic forms are generally associated with cell membranes via hydrophobic interactions at the N-terminus of the enzymes that are critical for activity. However, it has been reported recently that the soluble eukaryotic forms can be found in a yeast **(Nakayama *et al.*, 1996)** and in a plant **(Schuler, 1996)**.

1.7.7 Future for P450s

Future prospects for the study of the P450s seem interesting. Mammalian cell lines expressing different P450 isoforms are being developed as an advanced means of screening novel drugs. While overexpression of membranous proteins is notoriously difficult, great successes have been made for a number of mammalian P450s, through re-design of their N-terminal amino acids **(Fisher *et al.*, 1992)**. Biotechnological interest is high in the use of bacterial forms for degrading organic pollutants and there has already been major investment in the use of yeast P450s for steroid synthises. It has also been shown that electrical currents can be used to turn over P450 enzymes into a productive catalytic cycle **(Faulkner *et al.*, 1995)**.

With their wealth of roles and applications in biological and chemical fields, the P450s are certain to remain a major focus of interest in academic and pharmaceutical research, well into the next century. Their range of catalytic actions, their association with cellular membranes, and their current role in oxidative catalysis in the pharmaceutical industry, made this group of enzymes a suitable target for a study of the effect of solvents on biotransformations.

1.8 Rational for experimental programme

1.8.1 Solvents and membrane proteins

Solvents can affect the catalytic ability of proteins both by inhibition and by modification of global stability **(Klibanov 1986)**. They can also act as general anaesthetics by dissolving in to lipid membranes and affecting synaptic ion channels or their regulatory

systems. The anaesthetic potency of a molecule and its toxicity can be determined to some extent by its log P value.

Little work has been documented linking the toxicity or anaesthetic potency with actual concentrations of solvent that partitions into the membrane from the aqueous or gaseous phase. This reflects the difficulty in working with membrane bound proteins.
The solvent effects on progesterone 11α-hydroxylase activity in *Rhizopus stolonifer* have however been documented using aqueous two phase systems **(Ceen *et al.*, 1987; Osborne *et al.*, 1990)**. The progesterone 11α-hydroxylase system consists of two enzymes that function to hydroxylate the C-11 of progesterone. The microbial hydroxylation of progesterone carried out in an aqueous environment yields low productivity due to the low solubility of both the substrate and the product. **Ceen *et al.*, (1987)**, used an organic/aqueous two phase system to increase the concentrations of the substrates and the products, but this had an adverse effect on the activity of *Aspergillus ochraceous*. The use of long chain fatty acids however increased the stability and productivity over conventional aqueous media.
Osborne (1990) used *Rhizopus stolonifer* as a packed bed bioreactor and presented a detailed study of the factors that caused the loss of activity. They showed a high correlation between the loss of activity and the concentration of organic solvent that partitioned into the cell membranes as calculated by log P values. However, the initial effects of solvents on the rate of the hydroxylation were not measured. Inhibition of the reaction was recorded after a 24 hr incubation period. The distinction between inhibition of the hydroxylation and the stability of the fungi was not drawn.

1.8.1.1 The volume occupancy hypothesis

By modification of the Collander equation, they were able to show that the total loss of activity occurs at a single critical membrane solvent concentration irrespective of the type of organic solvent. Organic solvents unable to achieve this critical membrane concentration were shown to allow the retention of hydroxylase activity at saturating aqueous phase concentrations. However, with the development of a discrete second organic phase loss of activity occurred through so termed 'phase' effects **(Osborne, 1990)**. If the effects of the second phase were removed then this hypothesis links well with the

low solubility theory to explain the molecular 'cut off' effect in anaesthesia **(Pringle *et al.*, 1981)**. What all 'cut off' molecules have in common is a low solubility in the aqueous phase. So that even in equilibrium it is impossible, in principle, to deliver enough drug to the site. Also, the final equilibrium concentration at the site must be independent of the path. So that even if in theory the equilibrium concentration of solvent in the membrane is high enough the rate of the transfer may be limiting so that in practice the solvent is inactive as cellular toxin or anaesthetic. This hypothesis in relation to anaesthesia is extremely similar to the hypothesis described some time later by **Osborne, 1990.**

1.8.1.2 How do solvents deactivate membrane proteins?

Membrane proteins such as P450 hydroxylase enzymes were shown to be inhibited at certain volume occupancy by calculation from log P values **(Ceen *et al.*, 1987; Osborne *et al.*, 1990)**. However, no exact experimental determination of solvent concentrations associated with the membrane was carried out. In this study, a method to determine experimentally the exact concentration of solvent associated with the membrane and the inhibition of membrane proteins with actual concentrations of solvent that is intimate with protein and membrane will be developed.

To test the hypothesis an accurate measurement of solvent that is associated with the membrane phase is required. In the next section, the problems with this measurement and solutions are described.

1.8.2 Determination of Solvent Concentration

There is no accurate and reliable method for directly measuring solvent concentrations associated with biological membranes. However, methods for the routine detection and measurement of solvents in aqueous solutions are available. If we add a known volume of solvents to a mixture of membrane and water, and we have a method of measuring the concentration of solvent in the aqueous phase then by difference we can determine the concentration of solvent associated with the membrane.

1.8.2.1 Methods of aqueous solvent detection

Traditional methods include both gas chromatography and radio labelled solvent **(Sallee, 1978)**. Recent developments in the field of optical biosensors have produced detection methods for methanol in hydrocarbon mixtures **(Hubert *et al.*, 1995)**.

Both traditional and current techniques do not distinguish between solvent that is associated with the organic phase. These techniques rely on the total separation of membrane from the aqueous phase. This is technically difficult and failure to complete the process of separation results in high overestimation of the concentration of solvent, since hydrophobic organic molecules will tend to partition into the membrane. The presence of very small amounts of water-soluble impurities can produce large overestimates of solvent concentrations.

1.8.2.2 Luciferase as a method of solvent detection

Franks and Lieb investigated the hypothesis that anaesthetics act directly on membrane proteins and that the regulation is not mediated by the cellular membranes **(Franks & Lieb, 1982)**. They used purified luciferase from the North American firefly *P. pyralis* **(Franks & Lieb, 1984)**. In their studies they employed the luciferase enzyme to report on the solvent of the aqueous phase. They showed that the concentration of solvents needed to inhibit the enzyme by 50 % was of the same order as the concentration required to anaesthetise animals. They also reported that luciferase was unaffected by cell membranes **(Franks & Lieb 1986)**. The inhibition of the bioluminescent light reaction could be used to measure partitioning of alcohols into lipid bilayers.

1.8.2.3 Bioluminescence as a solvent detection method

When luciferase and D-luciferin react in the presence of oxygen ATP and magnesium ions a flash of light is emitted. When exposed to various lipophilic solutes, in buffer or in equilibrium with membrane suspensions the flash response is inhibited. By measuring the inhibition with and without membrane Franks and Lieb were able to calculate the membrane /buffer partition coefficient directly knowing only the volumes of buffer and membrane and; the initial amount of solvent added to the suspension.

This is possible because luciferase unlike previous methods only reports on the solvent in the aqueous phase. The inhibition of luciferase enables the detection of aqueous solvent without the removal of membrane. Errors due to minute amount (<0.01%) of membrane therefore do not arise. Also because inhibition is proportional to lipid solubility **(Franks & Lieb, 1984)**, water-soluble impurities that overestimate the concentration in the radioactive tracer techniques do not give rise to errors **(Franks & Lieb, 1986)**.

1.8.2.4 The luciferase reaction

The luciferase enzyme from the North American firefly *Photinus pyralis* can be purified using affinity chromatography to the crystalline protein, free of the substrate luciferin. Firefly luciferase combines with luciferin (RMM 280) in the presence of ATP, Mg^{2+} and O_2, to give a photon of light, **(Figure 1-5)**. When the reactants are rapidly mixed, an initial burst of light is followed by a slow decay. The maximum rate of light production is sensitive to the presence of anaesthetics such that the decrease in the initial rate of reaction can be correlated with the concentration of solvent.

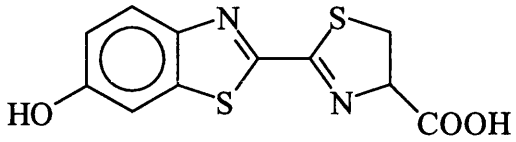
1.8.2.5 Inhibition of luciferase

In order to test the hypothesis that general anaesthetics act directly on protein, Franks and Lieb studied luciferase free from any lipid, so that any effects observed could be interpreted in terms of anaesthetic-protein interactions **Franks and Lieb (1984).** They showed that the maximum rate of light production was sensitive to the presence of anaesthetics and that there was a high correlation between the concentration of anaesthetic needed to inhibit the enzyme by 50 % (IC_{50}) and whole animal potencies (reciprocals of aqueous ED_{50} concentrations) over a 100,000 fold range.

1.8.3 A suitable membrane enzyme

For further examination of the molecular target of anaesthesia and the effects of solvents on membrane proteins, a membrane protein that can be readily assayed for its activity must be obtained. The membrane bound protein must not be part of a metabolic pathway since we would like to determine the effect of solvents on that protein only. If the product is metabolised a recording of the rate reaction will be difficult and the accuracy will be dependent on the relative rates of processes before and after the reaction of interest. A

membrane protein that metabolises excreted waste products is one option. The cytochrome P450 monooxygenase proteins are an example of drug metabolising enzymes. To separate the effect of the membrane on the reaction a soluble P450 for control is also required.



Luciferin (LH_2)

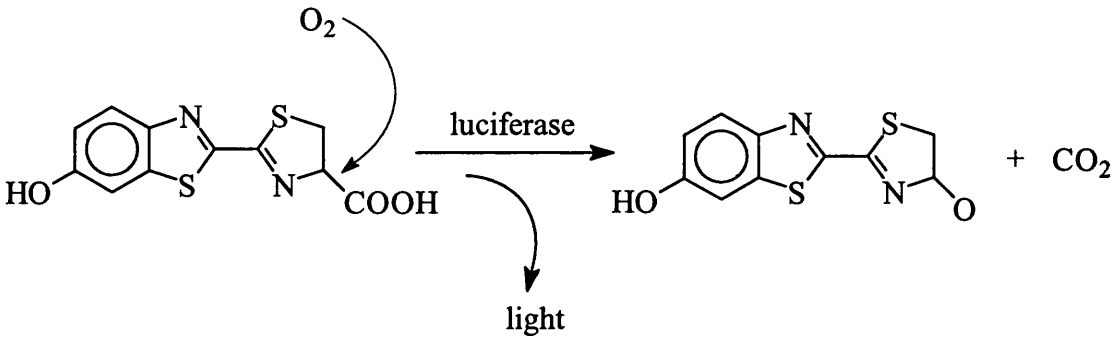


Figure 1-5 Luciferase catalysed oxidation of luciferin

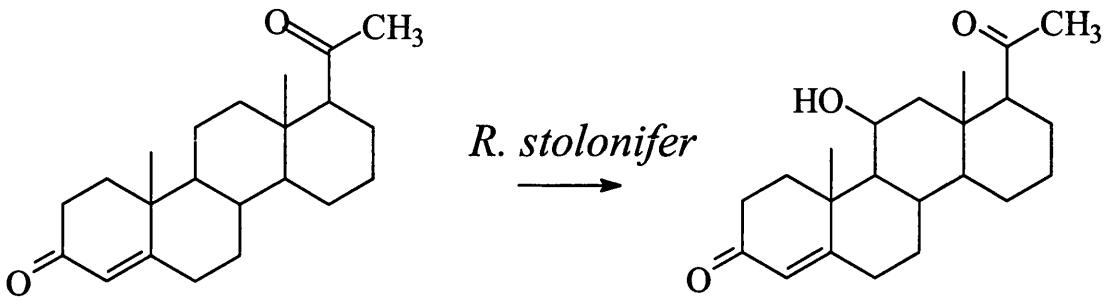
Methanol, ethanol, acetone, *n*-propanol, butanone, paraldehyde, diethyl ether, *n*-butanol, toluene, chloroform, *n*-hexanol, halothane, *n*-octanol, pentane, *n*-nonanol, hexane, and *n*-decanol were used as anaesthetics in the investigation.

1.8.3.1 Cytochrome P450 11α

Osborne (1990) investigated the effects of alcohols on the 11α-hydroxylase system from *Rhizopus stolonifer*. This steroid hydroxylating enzyme catalyses the conversion of progesterone to 11α-hydroxyprogesterone, **(Figure 1-6)**.

The growth and induction of the organism have been investigated **(Hanisch 1978, Osborne, 1990)**. Cell free extracts of *R. stolonifer* showed that the hydroxylase system is located in the microsomal fraction of the cytoplasm and involvement of the cytochrome P450 system is indicated in the reaction **(Osborne, 1990)**.

To test the hypothesis at least one membrane bound hydroxylase enzyme system is required. This can be obtained from *R. stolonifer* in the same way as **Osborne (1990)**.



Progesterone 11α-hydroxyprogesterone

Figure 1-6 Conversion of progesterone to 11α-hydroxyprogesterone by whole cells of *R. stolonifer.*

1.8.3.2 A soluble P450

To determine if the membrane plays a role in the interaction of solvent and protein we will require an enzyme of similar activity that is readily soluble and can be removed from all membranes in an active state. Cytochrome P450 BM3 from *Bacillus megaterium* satisfies these criteria and therefore we will look for a family of P450 type enzyme systems to investigate if the hypothesis holds for all or any of the membrane proteins. P450 enzyme systems are also extremely important enzymes since they function to hydroxylate a wide range of xenobiotic compounds.

2. Aims of the thesis

Previous work has demonstrated a correlation between Log P values and the inhibition of the membrane bound cytochrome P450 in *R. nigricans,* which hydroxylates the 11α portion of progesterone **(Osborne *et al,* 1992).**
The suggestion was made that this correlation in fact reflects a critical molar concentration of solvent in the membrane at which the enzyme is inhibited, and which is the same for any one of a homologous series of solvents **(Osborne *et al,* 1992).**

The correlation between an anaesthetic effect and a critical solvent concentration in biological membranes is one of long standing in the studies of general anaesthesia **(Seeman, 1972).** An assay developed for the use in these studies **(Franks and Leib, 1984)** is appropriate for measuring the aqueous solvent concentrations, which inhibit cytochrome P450 hydroxylases, and this is the starting point for this thesis, which aims.

1. To set up an assay to measure the equilibrium concentration of a solvent in the aqueous phase of a suspension of biological membranes derived from microbial cells. An assay based on the inhibition of luciferase, **(Franks and Leib, 1986)** is known to do this, but it must be modified to work with equipment available in the Dept. of Biochemical Engineering.

2. To compare the effect of organic solvents on a soluble and on a membrane bound cytochrome P450 hydroxylase in a manner which relates Log P value of the solvent to the actual concentration of solvent in the aqueous phase, rather than to the total concentration of solvent in the system. The soluble cytochrome P450 is derived from *B. megaterium* and it hydroxylates fatty acids. The membrane bound enzyme is the progesterone 11α hydroxylase derived from *R. stolonifer* (formally called *R. nigricans*).

3. To test the suggestion that a membrane bound enzyme is inhibited when the solvent concentration in the membrane exceeds a critical value, and that this is the primary effect in the link between the Log P or a solvent and its inhibition of enzyme activity.

4. To show how small differences between enzymes in any link between the Log P of a solvent and its inhibition might be reliably determined as the basis of developing incremental improvements in the resistance of enzymes to organic solvents.

2.1 Layout of Experimental sections

Section 3 describes the development of the luciferase assay of the aqueous solvent concentration.

Section 4 describes Isolation of P450 BM3 from *B. megaterium.*

Section 5 describes how P450 BM3 was isolated from *E. coli.*

Section 6 describes the effect of *n*-alcohols on the activity of cytochrome P450 BM3.

Section 7 investigates the hydroxylation of progesterone by whole cells of *R. stolonifer.*

Section 8 Inhibition of whole cells of *R. stolonifer* with *n*-alcohols.

Section 9 investigates the partitioning of solvents into whole cells of *R. stolonifer*

3. Development of a solvent biosensor based on firefly luciferase

The use of firefly bioluminescence to measure ATP derived from living biological material is now generally accepted and is the basis of numerous commercial products. The general principles of the light production and its measurement are well understood **(Section 1)**. This section of the thesis describes how the firefly light producing reaction, that is catalysed by the enzyme luciferase (EC 1.13.12.7), **(Equation 3-1)** was developed into a solvent biosensor in the presence of other biological material, using existing equipment available in the Dept. of Biochemical Eng.

$$\text{Luciferin} + O_2 + \text{ATP} \rightarrow\rightarrow \text{oxyluciferin} + \text{ADP} + \text{PPi} + CO_2 + \text{Light at 562 nm}$$

Equation 3-1 The bioluminescent reaction of luciferase

Firefly luciferase has been shown to report on only the solvent associated with the aqueous phase **(Franks & Lieb, 1984).** The flash kinetics associated with the luciferase-luciferin reaction **(Figure 3-1)** must be modified for an accurate and reproducible assay that does not change significantly with the time of measurement.

In its final form the solvent biosensor was stable, simple to use, and gave an accurate estimate of the aqueous concentration of a range of alkanes and primary alcohols.

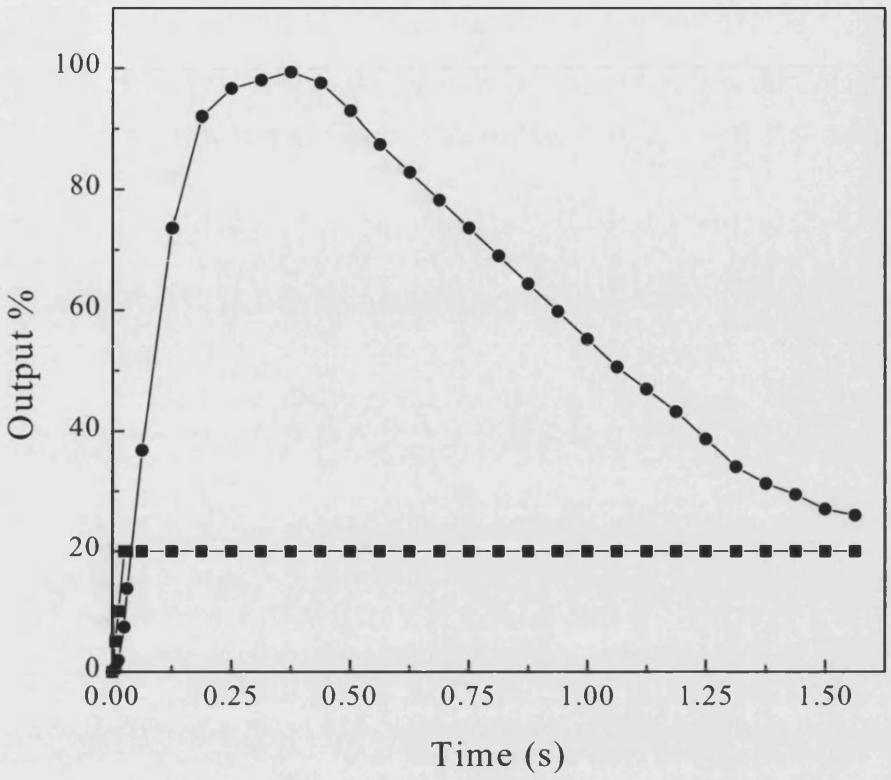


Figure 3-1 Comparison of flash emissions of the luciferase - luciferin reaction.

(●) **Franks & Lieb, 1984,** (■) the desired kinetics.

This chapter is divided into five sections.

Section 3.1 The selection of equipment suitable for measuring the light output from the luciferase assay.

Section 3.2 The measures used to stabilise the light output from the Enzymatix ATP kit.

Section 3.3 The measures used to stabilise the light output from commercially available luciferase and luciferin.

Section 3.4 Stabilisation of luciferase preparations.

Section 3.5 Use of the biosensor to measure aqueous concentrations of solvent.

3.1 Equipment for detecting light output

Two machines were available for detecting the light output from the luciferase reaction, the Perkin Elmer LS30 mulitmode spectrophotometer (Perkin Elmer, USA) and the Bio-orbit 1253 luminometer. Since at this stage the light output from the luciferase reaction was a brief flash rather than a steady glow (**Figure 3-1**), the machines were compared as fluorimeters, with flourescein as the assayed compound.

3.1.1 Perkin Elmer LS30

The multi-mode LS30 spectrophotometer has a flow cell as the detection window through which a pump transports the test sample. Although this provides reproducible results for the detection of fluorescein, it was found that it took 15 s for the test fluid to be pumped from the reaction container to the measuring window. For this reason the LS30 could not be used to measure a fast reaction. The detection system could only be operated once the test volume had entered the detection window and the pump had stopped.
Furthermore the minimum reaction volume required was 10 mL so that the cost of luciferin and luciferase would prohibit its use as a biosensor able to handle large numbers of samples. The LS30 was therefore ruled out as the useful detection system for luciferase and its development in measuring solvent concentrations.

3.1.2 Bio-Orbit 1253 luminometer

The Bio-Orbit 1253 is a photomultiplier tube (PMT) based luminometer. This design is more sensitive to light than spectrophotometry, so can detect lower light levels and samples of a lower concentration. The light tight measuring chamber is enclosed within a rotating tower that prevents exposure of the PMT to any external illumination. The sample (0.5 mL to 1.5 mL) is contained within a cuvette and so requires smaller amounts of test mixture than the LS30. Measurement of the light output is over a period of 1 s using manual control. The Bio-Orbit 1253 can also be connected to a PC for on line control. In this case using measurements over a period of 0.1 s are possible (LabVIEW®,

Section 3.5). The Bio-Orbit 1253 was able to detect the flourescein and the low sample volume and fast response conferred a useful advantage when compared with the LS30. For these reasons the Bio-Orbit 1235 was selected as the detection apparatus for all development work.

3.2 Stabilisation of light output from the Enzymatix ATP kit

3.2.1 The Enzymatix ATP kit reaction profile

An ATP detection system (Enzymatix, UK) was used to test the Bio-Orbit 1253 luminometer assay for response tailing and linearity of response to ATP. The Bio-orbit luminometer was found to have a linear response up to 4000 units **(Figure 3-2)**. The sensitivity to ATP can be decreased by a dilution of the luciferase-luciferin mixture. In this way, higher concentrations of ATP can be determined. However the brief flash of light from the reaction was not suitable for its use as a means of measuring aqueous concentrations of solvents, for which purpose a steady glow of light was considered more useful.

3.2.1.1 Effect of *n*-hexanol on the Enzymatix ATP kit

The effect of hexanol on the kit was tested to see if it would respond in the same way as reported with pure luciferase and luciferin **(Franks & Lieb, 1984)**. Hexanol at a concentration of 60 mM was found to activate the reaction to a small degree but cause a rapid decrease in the signal stability. At concentrations above 70 mM, hexanol reduced the initial maxima and the stability of the signal **(Figure 3-3)**.

The results confirm that alcohols function as inhibitors of the reaction at high concentrations as documented **(Franks & Lieb, 1984)**, but at low concentrations, promote the reaction. This has not been reported. When hexanol was added to a concentration of 250 mM the stability of the signal improved with a decay rate of less than 10 % measured per minute. However since the purpose of developing the luciferase assay is as a measure of solvent concentration it is not appropriate to use hexanol or any other solvent as a means of stabilising the light output. On the other hand the varying light output at low

concentrations of hexanol, and in the control stresses the need for a stable light output if the system is to be used to measure solvent concentrations.

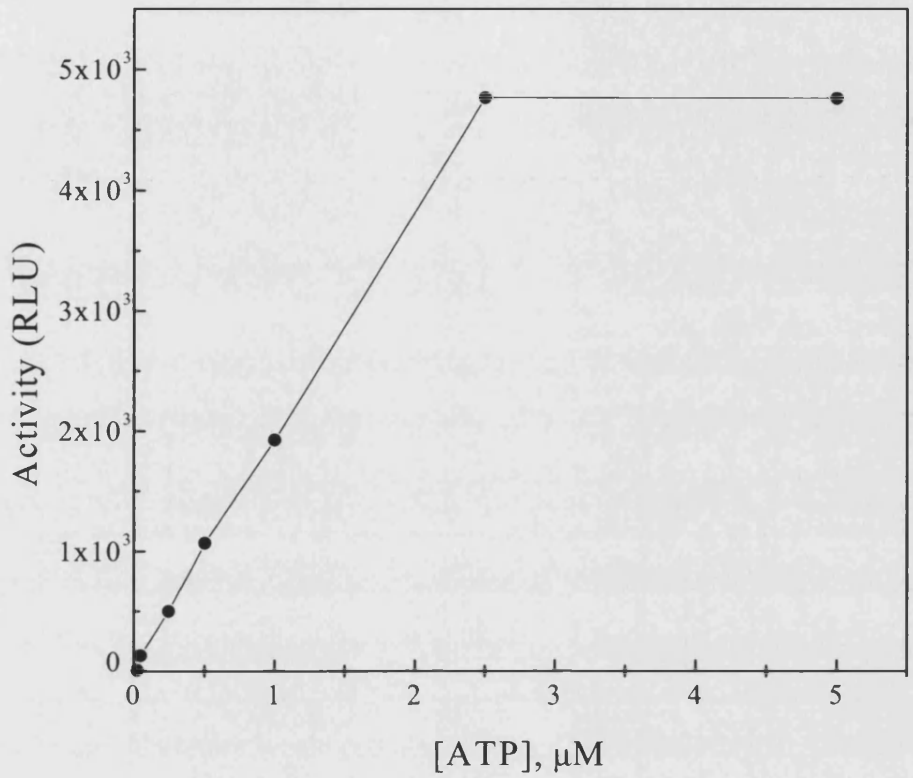


Figure 3-2 Response of the Enzymatix ATP detection kit to increasing concentrations of ATP.

The kit at a 16-fold dilution was tested with various concentrations of ATP. The reaction was monitored by luminometry (Bio-Orbit 1253 luminometer). The initial dilutions of material and measurements were performed as directed by the manufacturer. Activity was measured in Relative Light Units (RLU) and is a function of the photomultiplier tube mechanics.

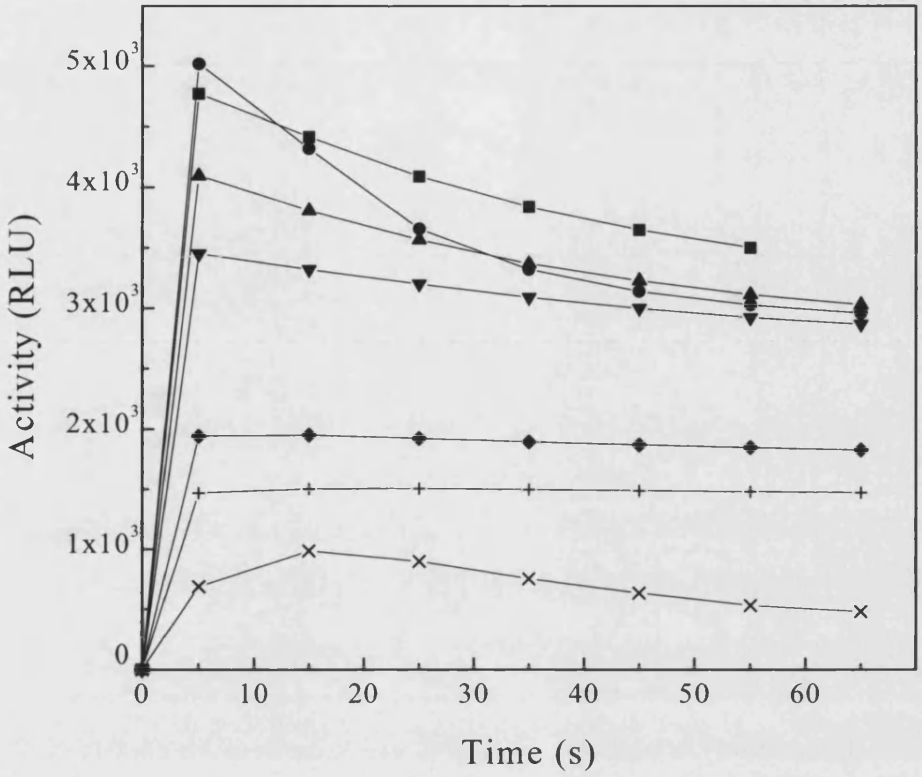


Figure 3-3 The effect of hexanol on the profile of the Enzymatix ATP detection kit.

Kit was diluted to 1/16 and the reaction was initiated with the addition of ATP to a final concentration of 330 μM. The reaction was monitored by luminometry using a Bio-Orbit 1253 luminometer. Concentration of hexanol, (●) control - no hexanol, (■) 60 mM, (▲) 80 mM, (▼) 125 mM, (♦) 185 mM, (+) 245 mM, (×) 490 mM.

3.2.2 Inhibition of the Enzymatix ATP kit with PP_i

PP_i is produced in the first reversible step of the reaction and so the use of PP_i as an inhibitor was tested. PPi inhibited the reaction at all concentrations and improved the signal stability particularly at concentrations in the region of 0.5 – 1 mM. However the actual signal response is reduced almost 10-fold **(Figure 3-4)**.

While the inhibitor could be used to improve the accuracy of ATP detection by reducing the error in measuring the initial activity, PPi cannot be used to stabilise the solvent detection system since further inhibition will result with the addition of alcohols. Further inhibition of light output leads to an unstable signal from the luminometer and an initial activity, which is dependent on time. Inhibitors that act at the binding site were therefore abandoned for other methods of reducing the signal decay rate.

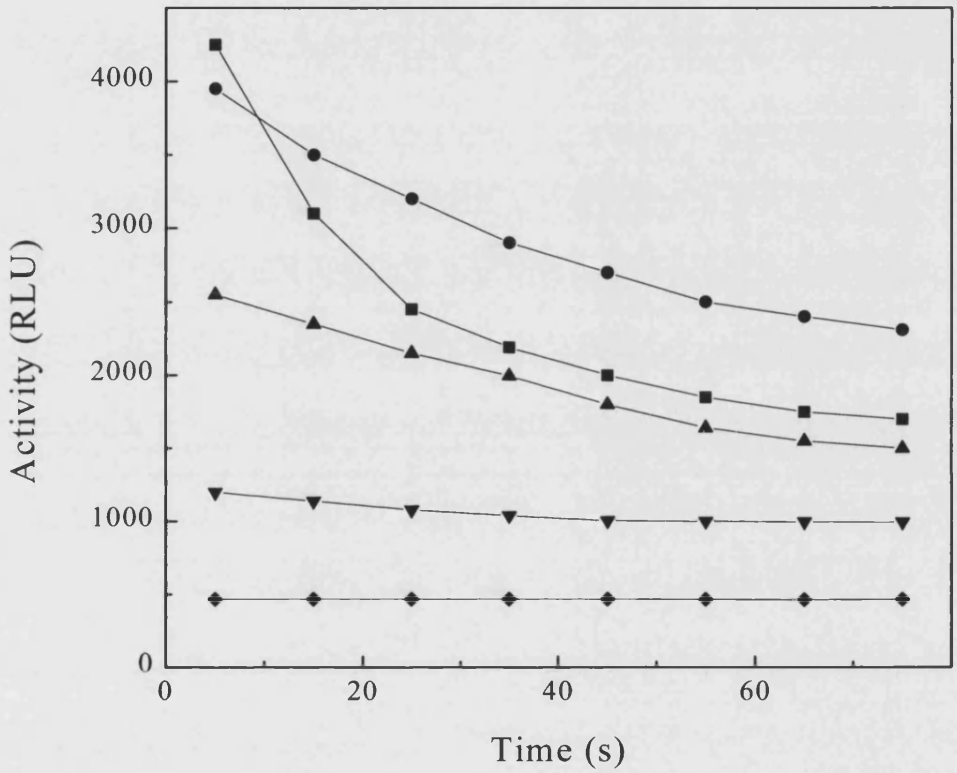


Figure 3-4 The effect of inorganic phosphate on the luciferase reaction profile.

The reaction was monitored by luminometry (Bio-Orbit 1253 luminometer).

Concentrations of PPi used: (■) control, (●) 50 μM, (▲) 100 μM, (▼) 500 μM, (♦) 1 mM.

3.3 Stabilisation of light output from commercially available luciferase and luciferin

The effect of the concentration of either luciferase or luciferin on the reaction profile could not be investigated using the ATP kits either from Enzymatix or from Sigma since both substrate and enzyme are premixed. Details of the components in the Sigma kit are shown in Table 3.1 and are typical. Data from this ATP kit are not shown, but they were similar to results obtained from the Enzymatix kit. To allow a better investigation of the assay, the components of the reaction were obtained from the supplier. The reaction was characterised starting from published data (Franks and Lieb, 1985).

Component	Concentration
Luciferase (firefly)	0.04 mg/mL
Luciferin	0.26 mM
HAS	4 mg/mL
$MgSO_4$	19.5 mM
EDTA	1.95 mM
Glycine salts	0.195 M
Tris	0.325 mM

Table 3-1 Constituents of ATP detection system.
Product No. L0633 as supplied by the Sigma chemical Company and diluted to a final concentration of 20 mg of solids to mL of water.

3.3.1 Effect of luciferin ind luciferase concentration

If the luciferin (0.1 μM), and luciferase (50 μg/mL), were mixed for 5 mins before being diluted with gly-gly buffer and $MgSO_4$ the light output rose very slowly **(Figure 3.6).** A comparison, with **Figures 3.4 and 3.5** shows that the maximum light intensity was very low.

In contrast when high concentrations of luciferase (62.5 - 125 μg/mL) are added to 2.5 μM luciferin there is a flash of light, which decays rapidly. Only if the luciferase concentration is low (12.5 μg/mL) is the glow of light stable, although it is much reduced in intensity **(Figure 3.6).** However even at these low concentrations of luciferase there is an initial burst of light before the steady glow begins unless the luciferin concentration is also reduced to a very low level (0.125 μM) **(Figure 3.7)**.

Although the latter conditions give a low light output it is easily measured in the Bio-Orbit luminometer and these conditions, (0.125 μM luciferin, 12.5 μg/mL luciferase), formed the basis of the standard assay **(Figure 3.7)**.

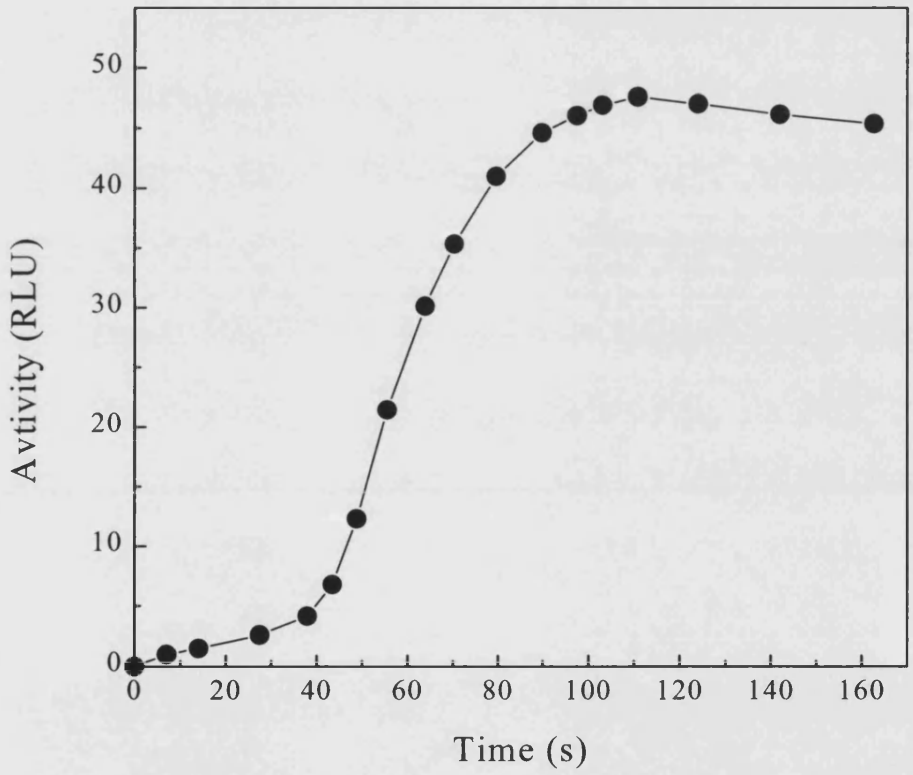


Figure 3-5 The effect of premixing luciferase and luciferin on the kinetics of the reaction.

The reaction was monitored by luminometry (Bio-Orbit 1253 luminometer). Luciferin (0.1 μM), and luciferase (50 μg/mL), were mixed for 5 mins before ATP and other reagents were added.

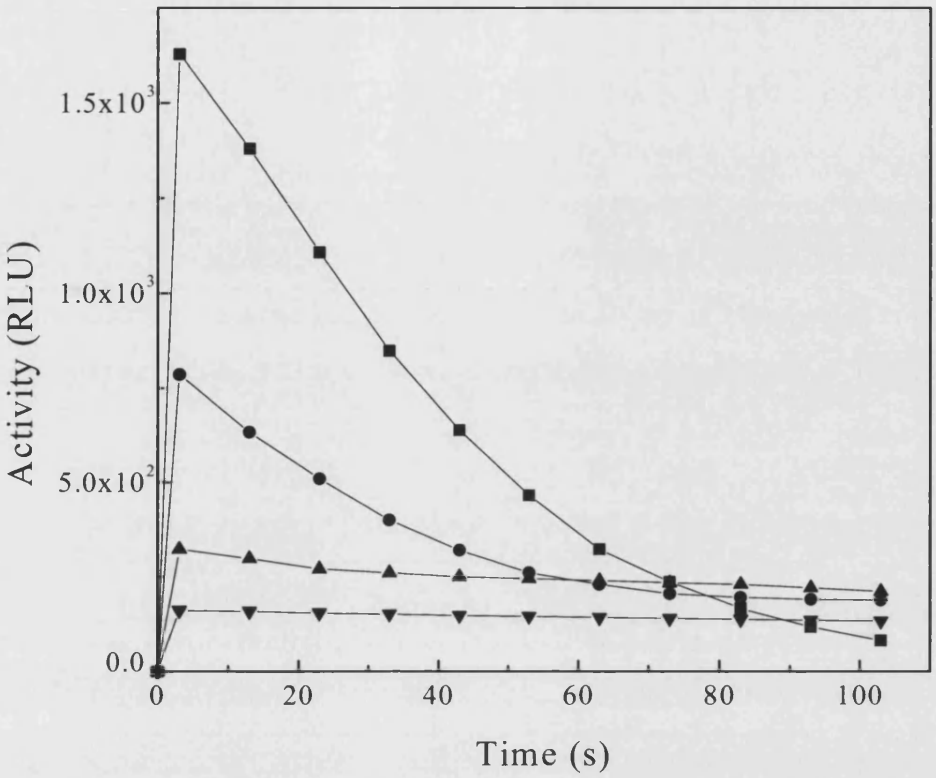


Figure 3-6 The effect of the luciferase concentration on the reaction profile. Luciferase (100 μL) up to a concentration of 1 mg/mL added to 800 μL of reaction mixture containing 25 mM gly-gly, 8.75 mM $MgSO_4$, 2.5 μM luciferin, and 625 μM ATP. Plot of activity vs. [luciferase] is a straight line. Concentration of luciferase, (■) 125 μg/mL,(●) 62.5 μg/mL, (▲) 25 μg/mL (▼)12.5 μg/mL

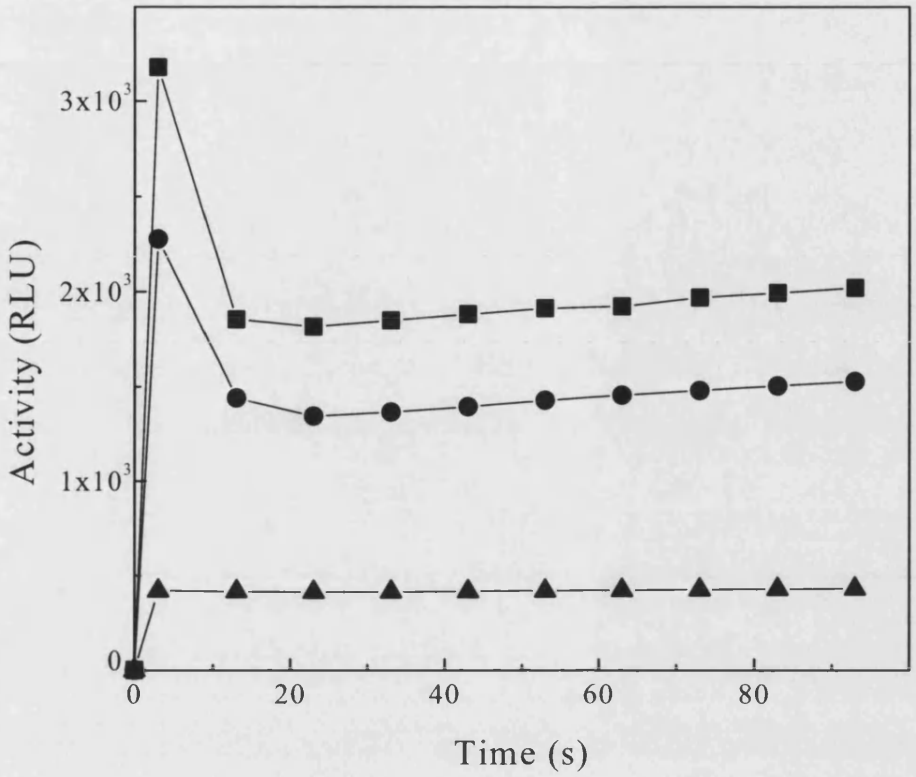


Figure 3-7 The effect of luciferin concentrations on the reaction profile.
Luciferin was added to a concentration of 2.5 μM added to 800 μL of reaction mixture containing gly-gly 25 mM, $MgSO_4$ 8.75 mM, luciferase 1.25 μg/mL, and ATP 625 μM. The reaction was monitored by luminometry (Bio-Orbit 1253 luminometer).
Concentration of luciferin in the assay: (■) 2.5 μM, (●) 1.25 μM, (▲) 0.125 μM.

3.3.2 Effect of ATP concentrations

The concentration of ATP in the final assay mixture was decreased and the effect of the lower concentration of ATP on the signal peak and the profile of the reaction was determined.

It was found that when lower concentrations of ATP were used the maximum activity decreased in a similar way to the Enxymatix ATP detection system used earlier **(Figure 3-**

4). However at the low luciferin and luciferase concentrations (0.125 μM and 12.5 μg/mL final concentrations), the concentration of ATP had little effect on the rate of signal decay so that the decay rate was within 10 % per minute **(Figure 3-8)**. This effect can be more clearly seen when the initial activity is scaled to an initial activity of 100 % **(Figure 3-10)**. An intermediate concentration of ATP (625 μM) was chosen for subsequent experiments.

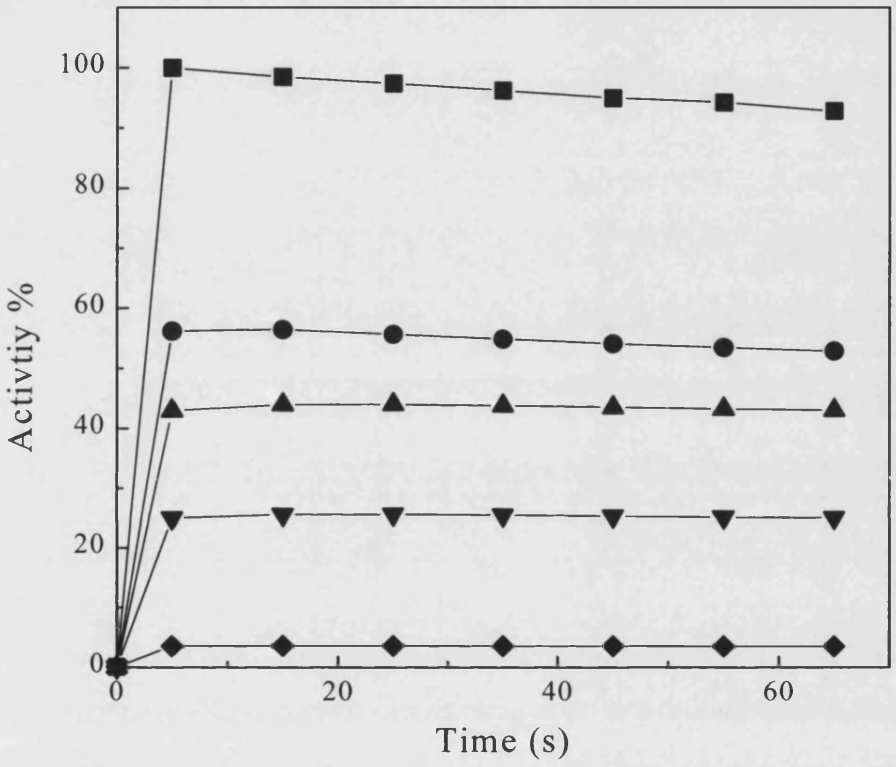


Figure 3-8 The Effect of ATP concentration of light output.

Initial output at 1.25mM ATP taken as 100%.

Data used is as for Figure 3-7 with 0.125 μM and 12.5 μg luciferase The graph shows the effect of ATP concentration on the rate of signal decay. Concentration of ATP, (■) 1.25 mM, (●) 630 μM, (▲) 500 μM, (▼) 250 μM, (♦) 50 μM.

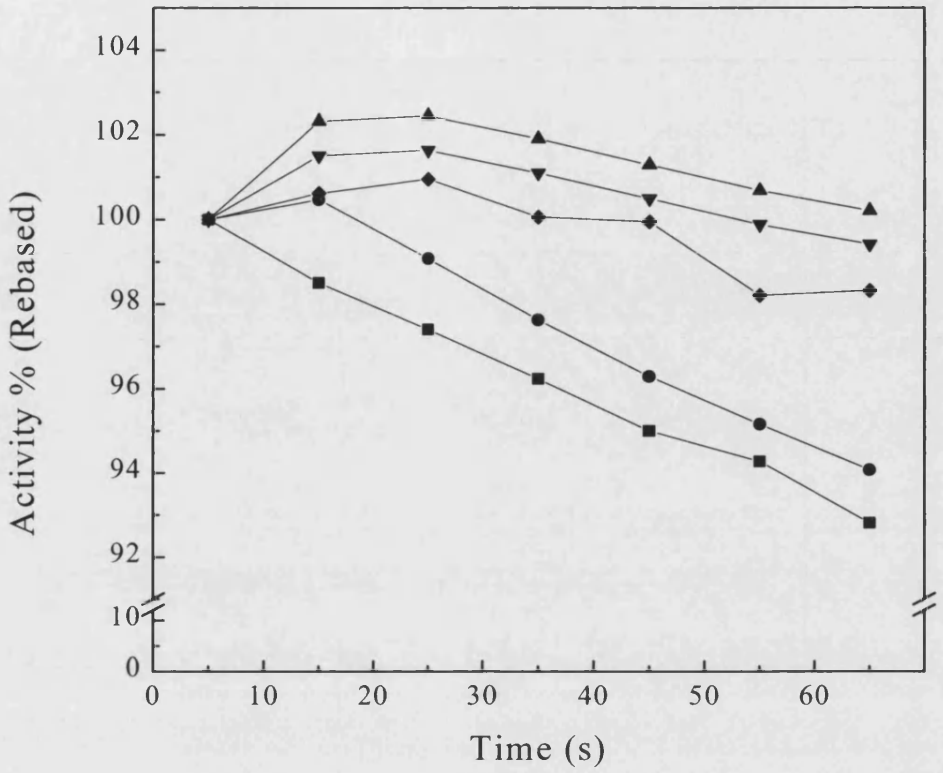


Figure 3-9 The effect of the ATP concentration on the signal stability.

Concentration of ATP, (■) 1.25 mM, (●) 630 μM, (▲) 500 μM (▼) 250μM, (♦) 50μM.

3.4 Improving the stability of Firefly Luciferase by Medium Engineering

3.4.1 Introduction

The low stability of luciferase in aqueous solvents can lead to errors in the determination of ATP concentrations and in the measurement of concentration of solvents in the aqueous phase. The stability was enhanced by varying the buffer type, ionic strength and with additives including sorbitol (D-glucitol) and glucose.

Both luciferin and luciferase are unstable at room temperature and prone to damage when freeze-dried. The activity of luciferase can decrease during the assay period. Therefore the controls must be repeated frequently and quickly. Luciferin is readily oxidised to oxyluciferin. The action of an aqueous phase accelerates the oxidation so that luciferin is most stable in the dry form.

Freeze-drying, the use of sugars, modification of buffers and the concentration of enzyme will be tested to obtain a useful, highly stable enzyme preparation.

Luciferase and luciferin are required in a stable form in small aliquots that can be dissolved into gly-gly to give a working solution that can be used for a number of solvent assays.

3.4.2 The effect of the buffer concentration on the activity of luciferase

A range of gly-gly buffer solutions (12.5 mM to 2 M), were prepared at pH 7.8. Luciferase was added to a final concentration of 1 mg/mL. The activity of luciferase was measured at the different buffer concentrations.

At high buffer concentrations the activity decreased and above 100 mM the activity recorded was lower than the control (12.5 mM) suggesting that the reaction is inhibited at high ionic strength solutions **(Figure 3-10).** When the buffer concentration decreased the activity increased. A maximum rate of activity was noted at 30 mM, below this concentration the activity decreased and at 1.2 mM the activity was only 20 % of the maximum activity.

3.4.3 The effect of the buffer concentration on the stability of luciferase

The stability of luciferase was measured in 25 mM gly-gly and 1 M gly-gly. The solutions were stored at 4 °C and the activity recorded over a period of 2 hr. The 1 M solution was diluted to 25 mM and the activity measurements adjusted for the concentration of luciferase.

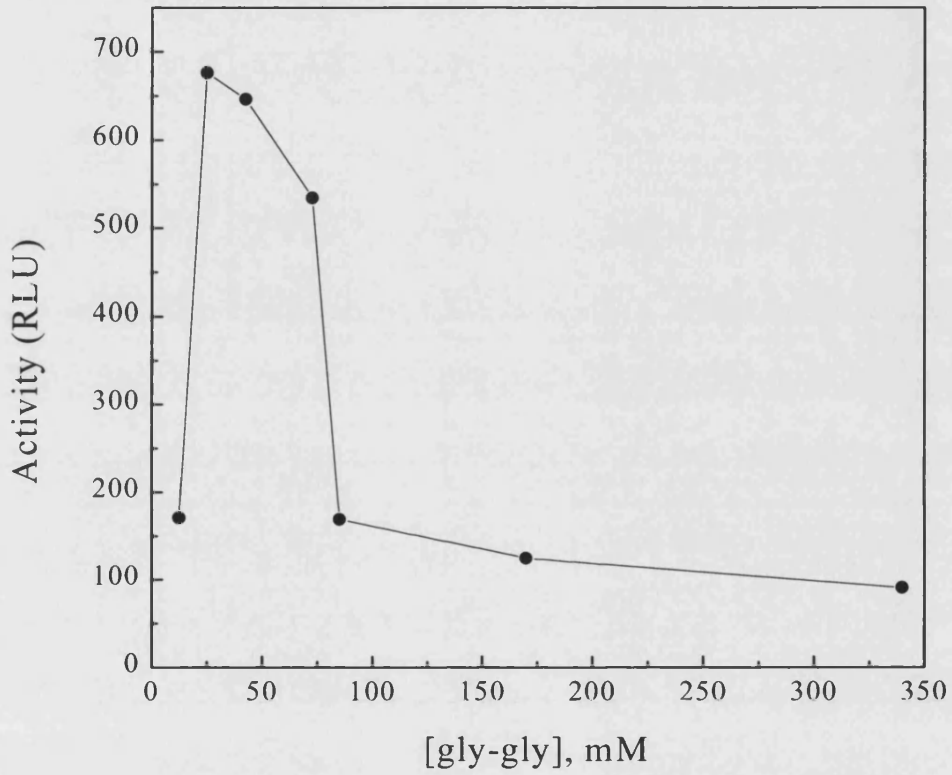


Figure 3-10 The activity of luciferase in high concentrations of gly-gly buffer, pH 7.8.
The final luciferase concentration was 1.25 mg/mL.

It was found that the activity of luciferase decreased in both samples over time. The rate of activity loss in the 1 M buffer was lower than for the 25 mM buffer **(Figure 3-11)**.
High buffer concentration may act to increase the conformational stability of luciferase. The luciferase enzyme is a dimer of two non-identical subunits. The ionic interactions of

the two subunits could be sensitive to the ionic strength of the media and this could be important in ensuring that luciferase exists as the dimer.

3.4.4 The effect of luciferase concentration on its stability

Luciferase solutions 1 mg/mL and 0.1 mg/mL in 25 mM gly-gly were stored at 4 °C and -20 °C. After one week, the activity of the solutions was tested.
At all concentrations and temperatures the activity is reduced **(Figure 3-12).** The loss of activity was minimised when the concentration of luciferase was high and the storage temperature was low.

3.4.5 The effect of BSA on stability of luciferase

1 mg/mL luciferase, in 1 M gly-gly, was prepared. BSA was added to the solution at concentrations of up to 8 mg/mL. The activity of the luciferase in BSA preparation was assayed after incubation at 4 °C for 35 min.

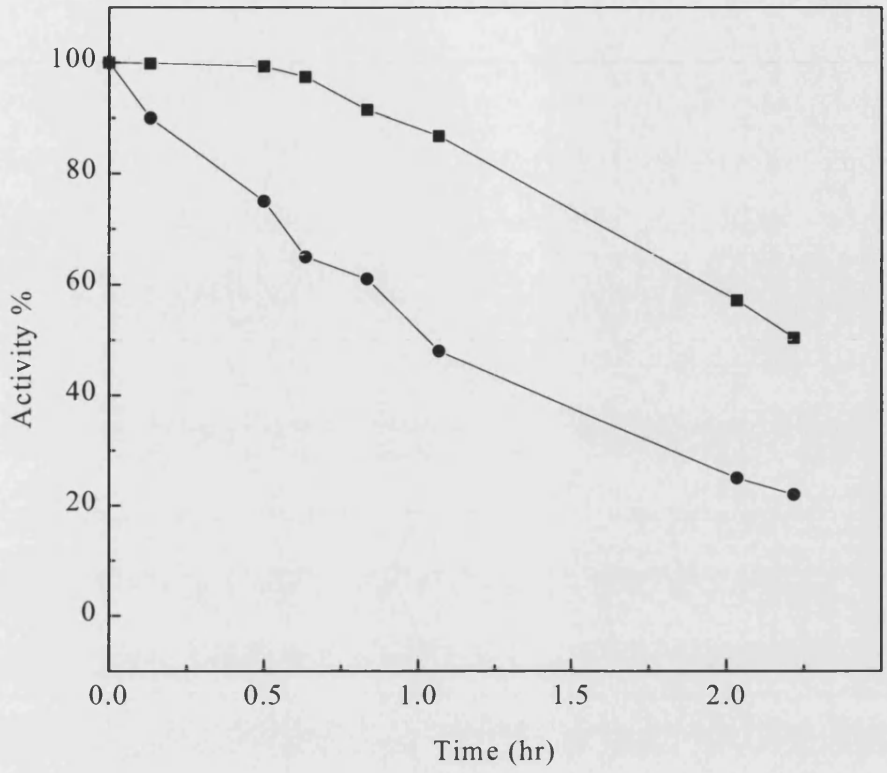


Figure 3-11 Effect of buffer concentration on the stability of 0.1 mg/mL luciferase stored of various times at 4°C in gly-gly buffer. In all cases the concentration of gly-gly is reduced to 25 mM for activity assays (concentration of gly-gly buffer during storage phase: (●) 1 M, (■) 25 mM.

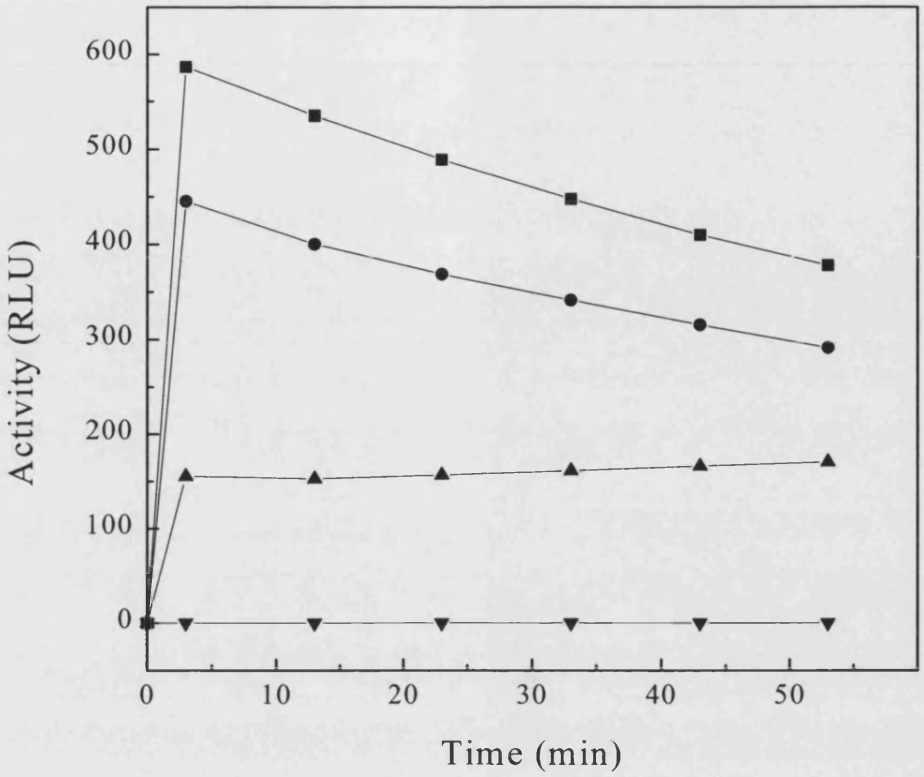


Figure 3-12 Effect of storing high concentrations of luciferase at -20 °C and +4 °C. Storage conditions (concentration of luciferase and temperature): (■) 1.0 mg/mL Control, (●) 1.0 mg/mL -20 °C, (▲) 0.1 mg/mL -20 °C, (▼) 0.1 mg/mL, 4 °C. The control sample was prepared just before the assay was performed.

It was found that concentrations of BSA lower than 3 mg/mL increased the initial activity. At concentrations of BSA higher than 3 mg/mL a 20 % loss of activity was recorded **(Figure 3-13)**.

At all concentrations of BSA, the loss in activity at 4 °C was reduced compared to controls without BSA. The action of BSA was thought to be non-specific reducing the contact at the surface of the plastic Eppendorf tubes or at the air/liquid interface where unfolding could occur. BSA however, was not used in the final preparation because of the requirement to remove extraneous sources of protein from the system that might prove to be complicating factors when the action of solvents on luciferase is tested.

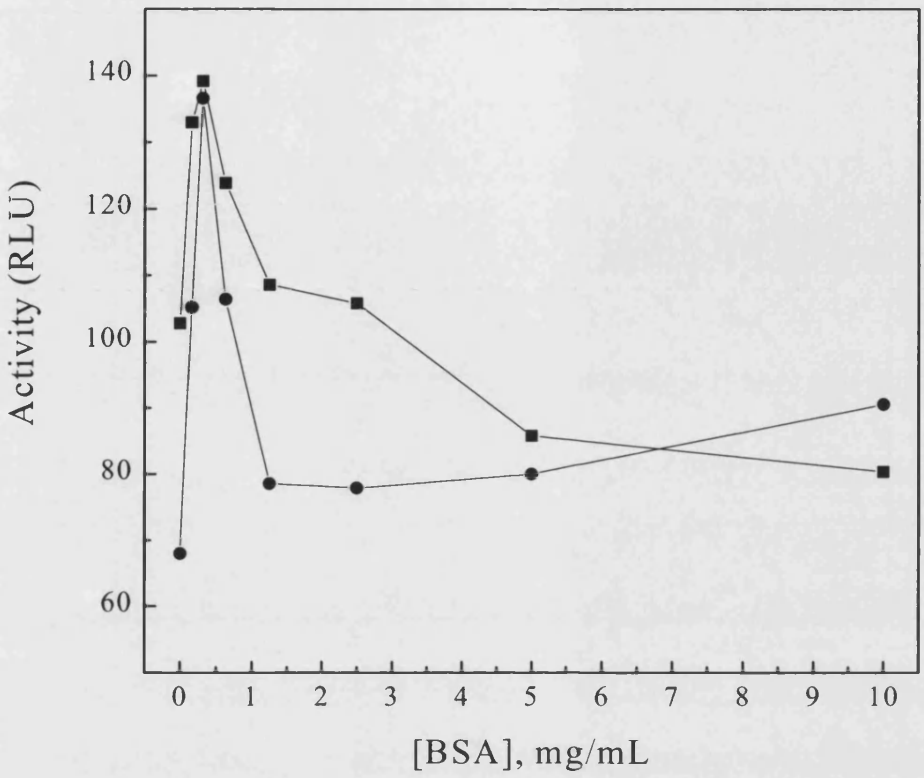


Figure 3-13 The effect of BSA on the stability of luciferase. BSA was added to a solution of luciferase in 25 mM gly-gly buffer to give the final concentrations indicated (up to 10 mg/mL). The enzyme solution was stored at 4°C for 5 or 35 min before assay. 10 µL of the enzyme solution was added to the normal reaction mixture. Storage times, (■) 5 min, (●) 35 min.

3.4.6 Effect of sorbitol in high molar strength gly-gly buffer

The use of sugars and polyhydric alcohols has been documented as an effective method of increasing the thermal stability of enzymes **(Devi & Rao, 1998).** The effect of sorbitol on the stability of a solution of luciferase in gly-gly buffer was tested.

With gentle heating at 40 °C, sorbitol was dissolved in 1 M gly-gly pH 7.8, to a final sorbitol concentration of 1 g/mL. At concentrations above 2 g/mL the solution was found

to behave as a glass at -20 °C, with very low flow characteristics **(Table 3-2)**. Luciferase was soluble in sorbitol at 1 g/mL, 2 M gly-gly, to at least 1 mg/mL.

[sorbitol] (g/mL)	[sorbitol] (M)	State at -20°C
0	0	Solid (ice)
0.25	1.40	Solid (ice)
0.50	2.80	Solid (ice)
0.75	4.10	Liquid
1.00	5.49	liquid
2.00	11.00	glass/liquid
3.75	20.59	glass

Table 3-2 States of sorbitol solutions in 2 M gly-gly, at -20 °C.
The solubility of luciferase was aided by the addition of the enzyme to sorbitol gly-gly at 30 °C.

Luciferase was dissolved in 1 g/mL sorbitol, 2 M gly-gly, to a concentration of 1 mg/mL. The effect of sorbitol on the activity luciferase reaction was then assayed.
The addition of 10 mM sorbitol, to the luciferase reaction mixture has no effect on the rate of reaction. Higher concentrations tend to reduce the activity. A 1.4 M sorbitol decreased the activity of the luciferase reaction to only 3 % when stored at 4 °C for 2 hours **(Figure 3-14)**.

3.4.7 Sorbitol as a protective agent in lyophilisation

As small aliquots of luciferase are required for the routine assay, the use of lyophilisation can be used to dehydrate small amounts of diluted luciferase. Up to 70 % of the activity can be lost in each freeze thaw cycle of unprotected luciferase. Sorbitol is an effective protector of enzyme conformation. Sorbitol may be used to increase the thermal stability of luciferase. This allows the enzyme to be stored at higher temperatures, such as room temperature.

Luciferase 1 mg, and sorbitol 1g were dissolved into 2 M gly-gly (1 mL) buffer pH 7.8. 100 µL aliquots of the luciferase solution were then added to 1 mL Eppendorf tubes. The solutions were then cooled to -20 °C and lyophilised (Speedvac SC100, Savant, USA) for 24 hr at a low dry rate. A solution of luciferase in gly-gly buffer was also lyophilised in the absence of sorbitol.

The freeze-dried powders were then reconstituted with water to a 1 mg/mL luciferase concentration and incubated at 20 °C. The activity of the reconstituted luciferase sorbitol mixture was then tested and compared with reconstituted enzyme without sorbitol and enzyme sorbitol mixture that had been stored at -20 °C for 24 hr.
The luciferase sorbitol mixture that had been stored at -20 °C for 24 hr. lost 40 % activity when compared to the reference. The luciferase sorbitol mixture that had been lyophilised lost 5 % activity

3.4.8 Discussion

High buffer concentrations were required for the retention of enzyme activity during storage. High concentrations of buffer were found to decrease the activity of the bioluminescence. Luciferase is a dimer held together by ionic interaction.

Luciferase may have been stabilised by the dissolution in high ionic strength 2 M gly-gly, buffer, pH 7.4. The addition of sorbitol to the reaction mixture limited the activity at high concentration but conferred an advantage to storage. At low concentrations such as in the diluted reaction mixture there was only a small effect on the activity. These two effects can be utilised in the formulation of luciferase to yield a stable enzyme that retains high stability in the diluted assay mixture.

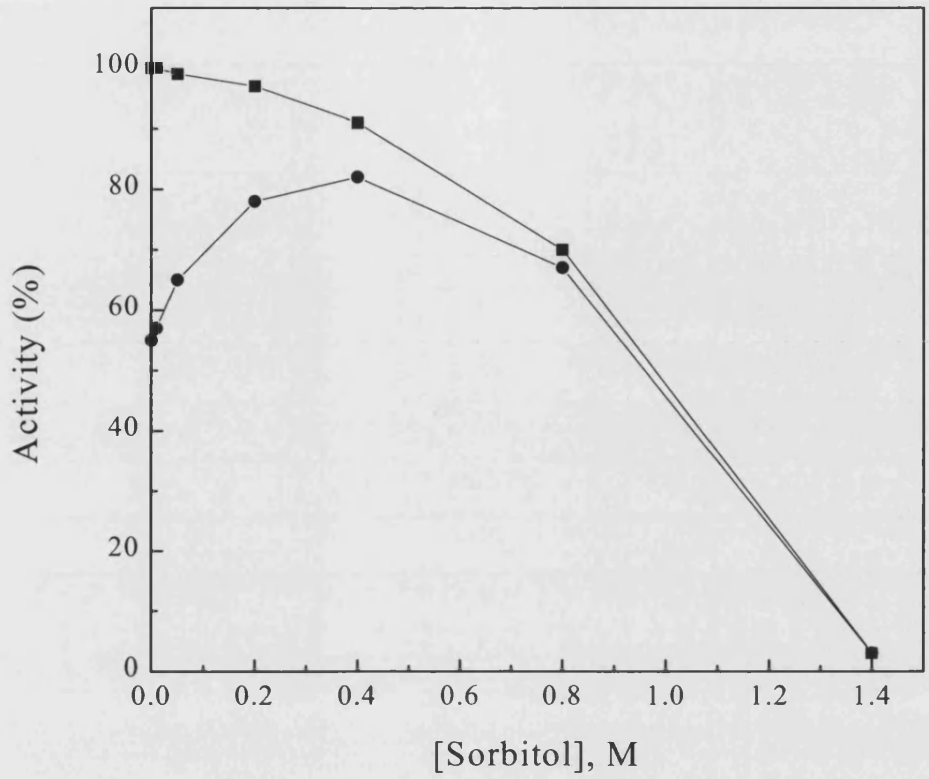


Figure 3-14 The effect of sorbitol on the stability of luciferase at 4 °C for 2 hours.

Assay conditions: 1.0 mg/mL Luciferase in 2 M gly-gly buffer pH 7.4, with increasing concentration of sorbitol. The activity was measured at the start of the reaction (■), and after 2 hr (●).

Luciferase contains two identical subunits, and it is likely that the high concentrations of gly-gly buffer and of the sorbitol influence their interaction. This could be tested with gel electrophoresis or gel chromatography. Whatever the cause both reagents do have a marked effect on the stability of solutions of luciferase. At 4°C 0.8 M sorbitol protects the luciferase for at least two hours **(Figure 3-14)**, but some activity is lost at -20°C even at sorbitol concentrations high enough to prevent freezing **(Table 3-2)**. On the other hand the solid obtained after freeze-drying from sorbitol solutions appears to be more stable. We have found the latter method useful in preparing the small quantities of luciferase

required for occasional ATP assays. A sample of luciferase, which is normally only stable in the dry state at -20°C appears to retain most of its activity over 24 hr at 22°C when sorbitol is present. Clearly high concentrations of both the gly-gly buffer and of the sorbitol are useful in preparing stable solutions of luciferase, and they should find some practical use in assays for ATP.

The effect of albumin (BSA) is more difficult to understand. This protein is known to bind small molecules, and it is possible that it interacts directly with luciferin itself as well as with the luciferase. It has been shown to stabilise the luciferase isolated from *Luciola mingrelica*. This may account for its dual action, of increasing the light intensity at low concentrations where it stabilises the enzyme, and of inhibiting the intensity at higher concentrations where it binds the substrate. This latter effect suggests that any protein, which binds luciferin, could affect assays for ATP.

3.5 Determination of solvent concentration using the biosensor

The use of the biosensor to determine the concentration of *n*-alkanes and *n*-alcohols in aqueous solution and in mixtures of membranes is discussed here. Also the use of a program for online control, data capture and data handling is described. The effect of online versus manual control on the accuracy of the data is also described.

Various anaesthetics can inhibit the conversion of luciferin to oxyluciferin by luciferase from the North American firefly. Media engineering has been used to successfully stabilise luciferase at 4 °C and modify the reaction profile from flash to steady state kinetics. The luciferase - luciferin reaction will be challenged by a series of *n*-alcohols from methanol to pentadecanol. The inhibition of the luciferase - luciferin reaction increases with the hydrophobicity, and the chain length of the solvent, **(Franks & Lieb, 1986)**. The concentration required to inhibit the reaction by 50 % (IC_{50}) will be recorded for each *n*-alcohol and *n*-alkane. In this way the reaction will be used as a biosensor for solvents in membrane/buffer mixtures.

3.5.1 Methods

Luciferase was taken from storage **(Section 3.2)** and revived with RO water (1 mL) to a 1 mg/mL luciferase concentration in 1 g/mL sorbitol, 1 M gly-gly, pH 7.8. 10 μM luciferin, 70 mM $MgSO_4$ and 10 mM ATP was also obtained.

Water (820 μL) was added to the 1.5 mL reaction vessel and then in strict order, luciferase (10 μL), luciferin (10 μL), and $MgSO_4$ (10 μL). The base line luminescence reading was then taken and ATP (50 μL) injected into the vessel. Changing the order of addition can have a dramatic effect on the reaction profile. For example adding luciferase to luciferin can cause a temporary inactivation of the enzyme. Readings were then taken every second.

For very hydrophobic solvents the IC_{50} point was very close to the solubility limit. It was for this reason the assay volume was increased from 800 μL **(Section 3.1)** to 900 μL.

For recording IC_{50} data, the assay was repeated, replacing a volume of water with the same volume of solvent. The volume of solvent added was varied such that a wide range of final solvent concentrations could be assayed. Luminescence data was recorded manually.

3.5.2 Manual data capture experiments

Luminescence data was taken manually by pressing the 'start' key at regular intervals. The values that appeared in the display window were recorded. However large errors were encountered when capturing the data manually, but the time of first measurement was reached from 3 sec (using manual operation) to ~0.1 sec (using on-line control).

3.5.3 Automated data capture experiments

A Graphical programming utility (LabVIEW®) was used to automate the control of the Bio-Orbit luminometer. This was required for fast control and data capture. A simple 6 frame sequence was used for the basis of the control package with data stored as a text (*.txt) file to be used by other graphical display applications (Microsoft Excel, Origin).

3.5.4 Results

Methanol was found to inhibit the reaction. Log IC_{50} value for the inhibition was 0.34 ± 0.02 M inhibits the reaction **(Figure 3-15)** also provides a 'standard curve' from which to

estimate the concentration of methanol in other aqueous samples. It is only necessary to measure the degree to which the unknown solution inhibits the luciferase for the concentration to be directly estimated from the figure. A series of 'standard curves' is easily constructed for the range of solvents under study. The advantage of the method is that only the aqueous concentration of the solvent affects the luciferase reaction; solvent, which is distributed in other phases of a complex mixture has no effect.

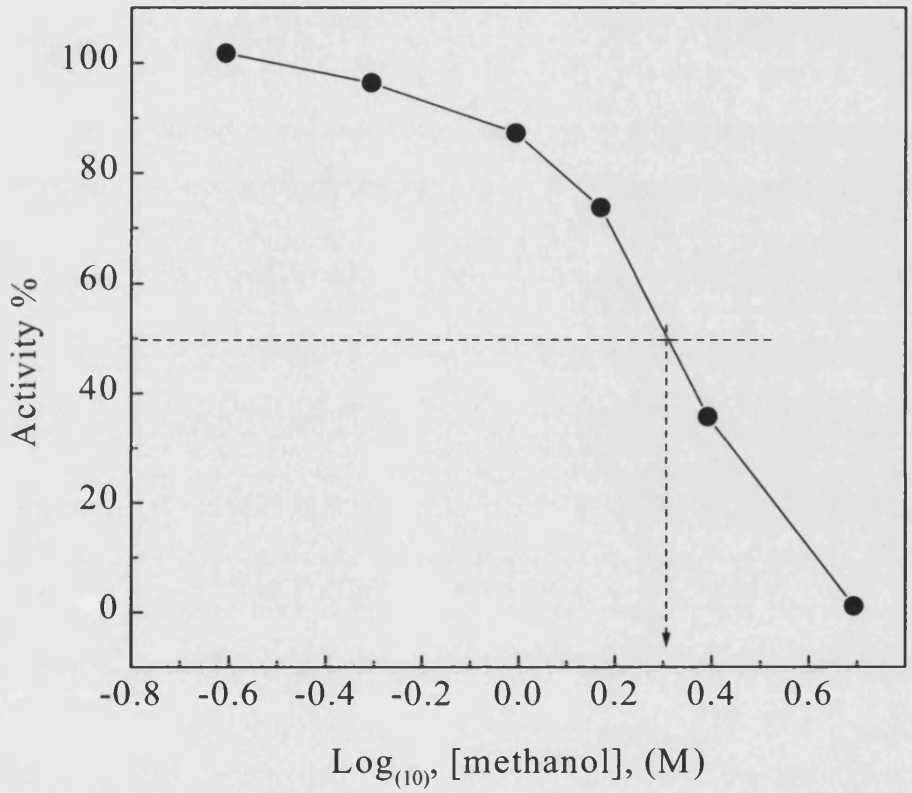


Figure 3-15 The effect of methanol on the biosensor.
Percentage of the initial activity remaining (●) on addition of alcohol is shown.

Methanol	2 M	0.30	octanol	510 μM	-3.29
Ethanol	360 mM	-0.44	nonanol	172 μM	-3.76
Propanol	330 mM	-0.48	decanol	44 μM	-4.36
Butanol	150 mM	-0.82	undecanol	7.7 μM	-5.11
Pentanol	29.4 mM	-1.53	dodecanol	2.81 μM	-5.55
Hexanol	11.9 mM	-1.92	tridecanol	0.25 μM	-6.60
Heptanol	1.77 mM	-2.75	tetradecanol	0.26 μM	-6.58

Table 3-3 The effect for alcohols in the series methanol to tetradecanol using LabVIEW®, (National Instruments™Texas, USA) to capture data.

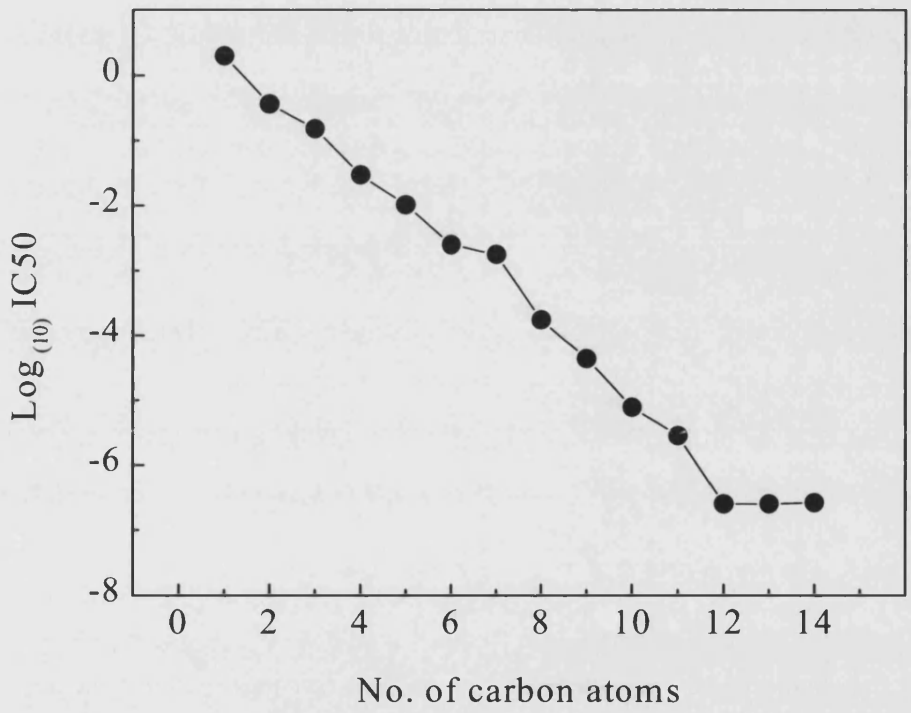


Figure 3-16 The IC_{50} of *n*-alcohols from methanol to tetradecanol on the luciferase in engineered media.

The data (●), was recorded using manual capture.

n-alkane	IC_{50}	Log (IC_{50})	*n*-alkanes	IC_{50}	Log (IC_{50})
pentane	125 μM	-3.9	decane	200 nM	-6.7
hexane	79 μM	-4.1	undecane	63 nM	-7.2
heptane	79 μM	-4.1	dodecane	16 nM	-7.8
octane	50 μM	-4.3	tridecane	12.6 nM	-7.9
nonane	3.1 μM	-5.5			

Table 3-4 Effect of *n*-alkanes on the luciferase reaction (automated data capture).

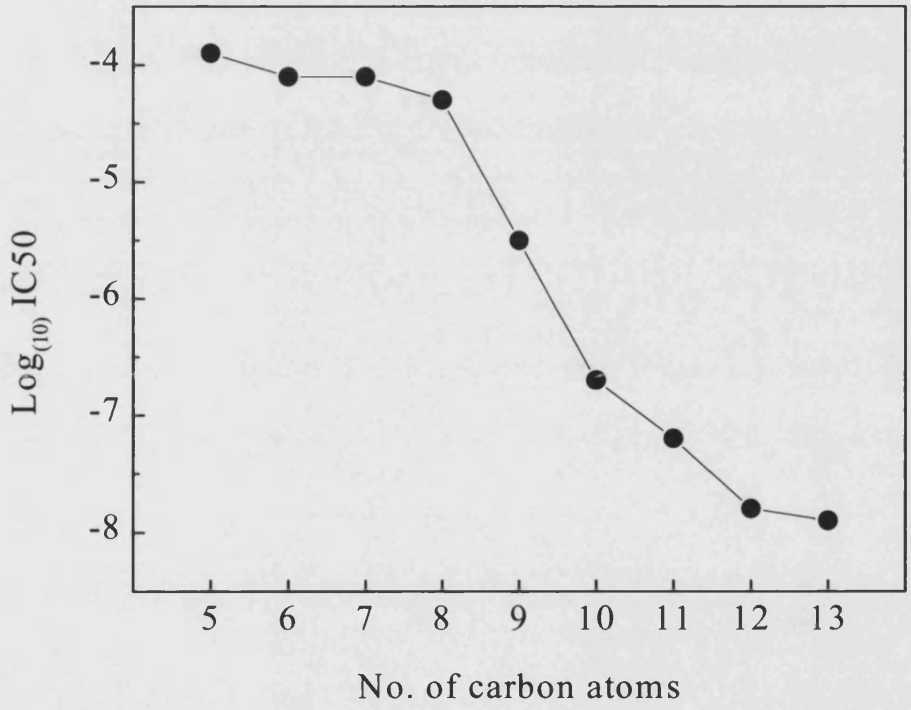


Figure 3-17 Effect of *n*-alkanes on the luciferase reaction (automated data capture)

The experiment was repeated several times for all alcohols from methanol to tetradecanol and the values of IC_{50} for each alcohol were calculated (**Table 3-3**).
n-alcohol's inhibited the luciferase reaction. The concentration of alcohol required to reduce the activity by 50% decreased with increasing chain length. Tetradecanol however, did not follow the trend. The concentration required to inhibit the enzyme by 50 % does not decrease and was similar to tridecanol. The *n*-alcohols with a carbon chain length greater than 14 could not reach the IC_{50} point because the solubility was lower than required.

The IC_{50} data for both primary alcohols **(Table 3-3),** and alkanes **(Table 3-4),** were lower than previously reported **(Franks & Lieb, 1985)** using manual data capture. Higher concentrations of solvent were required to give the same inhibition. This result can be explained by the fast kinetics of the reaction. Using manual control the first accurate reading that can be recorded is at 3 s after initiation of the reaction using a manual injection of ATP. If the initial activity is measured at 3 s then the measured activity is lower than the actual initial activity. The concentration required to reduce this activity will be lower than to reduce the actual activity by 50% (IC_{50}) The more accurate the reading of the initial activity the more accurate the IC_{50} data. Fast on-line data capture is required for accurate determination of inhibition data.

When the latter IC_{50} values for a range of alcohols and alkanes were plotted **(Figure 3-16 and 3-17),** the data was similar to the historical values **(Franks and Lieb, 1984).**

4. Isolation of P450 BM3 from *Bacillus megaterium*

The attempted expression of P450 BM3 from *B. megaterium* is documented in this section.

4.1 Abstract

B. megaterium ATCC 14581 was grown on complex and casamino acid salts media **(Black *et al.*, 1994)** to a scale of 2 L. Pentabarbital was added to the growing culture at mid-log to induce the production of Cytochrome P450 BM3. High cell densities of *B. megaterium* were obtained from cultures on complex media, but very low cell densities were observed when using casamino acid salts media. Several sources of casein were tested but neither spectrophotometry or light microscopy could obtain measurable biomass. Cells from complex media were harvested, ruptured by homogenisation and an attempt was made to purify P450 BM3 by affinity chromatography. P450 BM3 was not detected using spectrophotometric or catalytic techniques.

4.2 Introduction

The only well-characterised bacterial cytochrome P450 that belongs to class II is cytochrome P450 BM3 from *B. megaterium*. This soluble enzyme is unusual as it contains both a flavoprotein reductase and a P450 component in a single polypeptide chain of 1048 residues **(Narhi & Fulco 1987).** P450 BM3 is therefore catalytically self-sufficient in the mono-oxygenation of fatty acids requiring only NADPH and O_2 to function in the hydroxylation of long chain fatty acids. P450 BM3 is also unusual in that it is the only prokaryotic P450 known to resemble the mammalian microsomal P450 microoxygenases **(Black *et al.*, 1994).** After limited proteolysis with trypsin in the presence of substrate, the holoenzyme [molecular weight (RMM) 119kDa], is cleaved into (i) the NH_2 terminal (Heme) domain (RMM 55kDa), and (ii) the CO_2H terminal (flavin) reductase domain (RMM 66kDa), which contains FAD and FMN **(Li *et al.*, 1991).** Therefore, P450 BM3 resembles microsomal P450s, which requires FAD/FMN containing reductase for activity.

The heme (NH_2) domain incorporates a heme group and is capable of binding substrates and O_2, whereas the flavin (CO_2H) domain accepts electrons to the heme active site in an intramolecular electron transfer reaction. After trypsin digestion, the heme domain retains the ability to bind substrates and react with CO to give a characteristic peak at 450 nm. The flavin domain retains the NADPH dependent cytochrome *c* reductase activity, which can be used to monitor P450 activity. The monooxygenase activity Although the reported activity of the reconstituted system by different authors varies: **(Munro *et al.*, 1994),** reports an activity of only 0.3% measured by NADPH fatty acid consumption, whereas **(Boddupalli *et al.*, 1992),** reports an activity of 80% when measured by oxygen consumption.

Production of P450 BM3 (an inducible enzyme) from *B. megaterium* was investigated using various casamino-acid salts and complex media.

4.3 Materials

4.3.1 Source of *B. megaterium*

*B. megaterium (*ATCC 14581), was supplied by the American Type Culture Collection.

4.3.2 Solid media

Casamino acid salts media based on previous work **(Black *et al.*, 1994),** and a complex liquid media (Nutrient broth, Oxoid) was produced.

4.3.2.1 Casamino acid salts solid media

Three components **(Table 3-1)** of x2 concentration were autoclaved separately and then combined in the ratio of 98:1:1. The x2 salts media was then added in equal volume to 24 g/L agar technical (Oxoid) to give a final concentration of 12 g/L. Solid media was left to cool and later incubated at 30 °C for 5 days. Contaminated plates were discarded.

4.3.2.2 Complex solid media

Nutrient Agar plates were made to the manufactures specifications of 28 g/L agar (Oxoid Ltd.) in RO water. The solution was then autoclaved (120°C, 20 min) and 30 mL poured

into each petri dishes. Solid media was left to cool, then incubated at 30 °C for 5 days. Contaminated plates were discarded.

4.3.3 Liquid media

Casamino acid liquid media **(Black *et al.*, 1994)**, and a complex liquid media (Nutrient broth, Oxoid) were produced.

4.3.3.1 Casamino acid salts liquid media

Three components **(Table 4-1)** were autoclaved separately and then combined in the ratio of 98:1:1.

Part	Reagent	Molecular formula	Conc
1	Potassium nitrate	KNO_3	100 mM
	Potassium sulfate	K_2SO_4	1.0 mM
	Monobasic potassium phosphate	KH_2PO_4	50 mM
	Magnesium sulfate heptahydrate	$MgSO_4$ $7H_20$	0.5 mM
	Manganous sulfate tetrahydrate	$MnSO_4$ H_2O	10 µM
	Ferric sulfate heptahydrate	$Fe_2(SO_4)_3$ $7H_2O$	33 µM
	zinc sulfate heptahydrate	$ZnSO_4$ $7H_2O$	50 µM
	casein hydroxylate	various types **(Table 4-2)**	5 g/L
	citric acid monohydrate	C_4H_8O $7H_2O$	4.17 mM
	Sodium hydroxide	NaOH	5 g/L
2	D-glucose	$C_6H_{12}O_6$	2.78 M
	calcium chloride dihydrate	$CaCl_2$ $2H_2O$	56.45 mM
3	ferric ammonium citrate		4 g/L

Table 4-1 Casamino acid salts liquid media composition.

Name	Supplier	Source	Total nitrogen (%)	Total NaCl (%)	Total amino Nitrogen (%)	Method Of Production
Casamino acids Technical	Difco	Bovine milk	7-10	25-35	n.a	acid hydrolysis
Hy-Case SF	Sigma	Bovine milk	13.8	0.4	n.a	acid hydrolysis
Hy-Case Amino	Sigma	Bovine milk	8.2	34.3	n.a.	acid hydrolysis
N-Z-Case Plus	Sigma	Bovine milk	13.3	n.a	6.6	

Table 4-2 Details of the casein hydroxylate types used.

4.3.3.2 Complex liquid media

Nutrient broth13 g/L. Components in a typical batch can be seen **(Table 4-3).**

Component	% (w/w)
'Lab Lemco' Powder	7.70
Yeast extract	15.4
Peptone	38.5
Sodium Chloride	38.5

Table 4-3 Composition of the components in nutrient broth, %(w/w).

4.3.4 Downstream processing buffers

Cells of *B. megaterium* were homogenised in wash buffer I (0.1 M potassium phosphate, 0.1 mM dithiotreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM EDTA, pH 7.4).

Wash buffer I contained components to reduce disulfides and prevent proteolytic degradation during cell breakage and purification.

Wash buffer II (0.5 M potassium phosphate, 0.1 mM dithiotreitol, 1 mM EDTA, pH 7.4) was used to increase the ionic strength of the environment of the affinity matrix to encourage the elution of components not bound to the matrix

Wash buffer III (0.5 M potassium phosphate, 0.1 mM dithiotreitol, 1 mM EDTA, 3 mM 2'-AMP, pH 7.4) wash used to remove specifically bound oxidoreductases added to the matrix.

P450 BM3 was eluted with the addition of elution buffer (potassium phosphate 0.5 M, dithiotreitol 0.1 mM, EDTA 1 mM, 2'-AMP 50 mM, pH 7.4) with a high concentration of the competitor AMP.

4.3.5 Spectrophotometry

UV spectrophotometric assays and absorbance readings were recorded (ATI Unicam UV2 UV/Vis photospectrometer). A water bath and immersion thermostat (VFK, Grant) provided temperature regulation.

4.3.6 Light microscopy

A light microscope (Olympus CH) was used for all microscopy work. Growing microorganisms were viewed (D Plan 100 oil optic) with an overall magnification of 1000x. Cedar wood oil was used to lubricate the lens and the cover plate.

4.3.7 Fermentation equipment and analysis

Fermentation was performed in a glass bioreactor (2.1 L Inceltech LH series 210). Analysis of fermentation exit gases was achieved using O_2 and CO_2 (Ingold) detectors. This information was passed on to data logging personal computers where values of the carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and the respiratory quotient (RQ) are calculated. Data logging for the gas analysis and pH was achieved using a Real Time - data acquisition system (RT-das). These logged information from individual sender units at 3min intervals. Temperature, agitator speed and pH data were also recorded using this system.

4.4 Methods

4.4.1 Maintenance of *B. megaterium*

13 g/L nutrient broth (3 mL) was added aseptically to the vial, mixed and transferred to a test tube with nutrient broth (5 mL). After 3 days of incubation at 25 °C growth was detected and a sample streaked on to nutrient agar plates. When heavy growth was seen individual colonies were inspected by light microscopy and then used to inoculate of nutrient broth (5 mL). Populations from this test tube were then stored at -20 °C in 20 % (v/v) glycerol. Frozen stock cultures were revived at 25 °C in nutrient broth and plated on to solid nutrient agar.

4.4.2 Spectrophotometric absorbance measurements

The biological mass (biomass) of the culture was determined by absorbance measurements at 670 nm (A_{670}) **(Figure 4-2)**. When the absorbance of the culture exceeded 0.7 the sample was diluted to within the range 0.3 to 0.7 absorbance units for accuracy.

4.4.3 Solid media growth

Samples of *B. megaterium* were removed from -20 °C and revived in liquid nutrient broth (5 mL) until germination was detected by A_{670} measurements and light microscopy (typically 16 hr). Cultures were transferred to solid media and incubated at 30 °C. After 2-3 days heavy growth was seen, plates were stored at 4 °C for 6 weeks.

4.4.4 Shake flask fermentation

Starter cultures were prepared by either inoculating nutrient broth or casamino acid salts medium (100 mL) in a 500 mL baffled shake flask with a single colony from a nutrient agar plate. Incubation was carried out for 16 hr on an orbital shaker (200 RPM, 37 °C).

4.4.5 2 L Fermentation

Part 1: (1.96 L) of the casamino acid salts medium was sterilised *in situ* by heating with steam to 121 °C at 1.1 bar pressure for 20 min. At this stage the medium was allowed to

cool to 37 °C. Parts 2: (20 mL) and 3: (20 mL) was then added and the temperature maintained at this level. The inoculum (100 mL), from a 500 mL shake flask, was transferred to the 2 L vessel to give a final volume of 2.2 L and growth was allowed to proceed. Temperature was controlled at 37 °C while the pH was not controlled. Agitation rate was set at 800 RPM to give good dispersion of air into the vessel at a rate of 5 L/min. Growth was followed and harvest occurred when the DOT began to rise after induction.

4.4.6 Induction of P450 BM3

B. megaterium contains the cytochrome P450 BM3 and is inducible by barbiturate drugs **(Shaw & Fulco, 1983).** It has been demonstrated that a transcriptional repressor, termed BM3R1, is directly involved in mediating this response **(Shaw & Fulco, 1993).** BM3R1 binds to an operator upstream of the P450 BM3 operon and prevents transcription. Barbiturate drugs **(Shaw & Fulco, 1993)** and peroxisome proliforators **(English *et al.*, 1994)** interact with the BM3R1 and dissociate it from its operator allowing transcription to occur.

A aqueous solution of 0.5 g/L Sodium pentabarbital **(Figure 4-1)** in was added to the fermentation to achieve a final concentration of 5 mM at mid- log phase, shown by rapid increase of A_{670} values with time **(Black, 1994).**

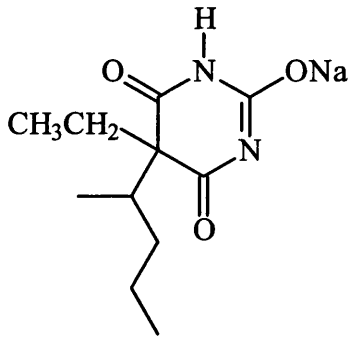


Figure 4-1 Representation of sodium Pentabarbital

4.4.7 Cell Harvest

Cells were harvested at early stationary phase shown by an increase in DOT following (up to 3 hr) induction. Harvested cells were concentrated to a cell paste by centrifugation (10,000 *g*, 20 min., 4 °C). The supernatant was poured away and the compacted cells suspended in wash buffer I, and centrifuged again.

4.4.8 Cell rupture by homogenisation

Clean cell pellet was suspended in the minimum amount of wash buffer I to a volume of 40 mL. Cells were disrupted by homogenisation (micron Lab40, APV), releasing intracellular components. Adequate disruption of cells was provided by one pass at 4 °C, 9,000 p.s.i.

4.4.9 Cell free extract

The samples of the cell homogenate were centrifuged (40,000*g*, 30 min. 4 °C). The supernatant fraction was poured into a chilled vessel and frozen at -70 °C until use.

4.4.10 Affinity Chromatography

Thawed cell free extract was then centrifuged (40,000 *g* 30 min., 4 °C) to remove any precipitate. Five column volumes of extract were loaded on to 2',5'-ADP agarose matrix, and then flushed with Reconstitution, wash and elution buffer **(Table 4-4)**.

Buffer	Column Volumes
Cell free extract	5
Reconstitution	10
Wash I	6
Wash II	5
Elution	7

Table 4-4 Elution protocol for P450 BM3

4.4.11 Assays

4.4.11.1 Reduction of cytochrome P450 BM3 with dithionite

Samples of wash buffers and elution buffers that had been passed through the column were assayed for the presence of cytochrome P450 by observing the reduced dithionite spectra. To 1 mL of sample, a small quantity (~20 mg) of solid sodium dithionite was added and CO was bubbled through for 1 min. the absorbance at 450 nm (A_{450}) was taken against a reference of wash buffer.

4.4.11.2 Catalytic assay for P450 BM3

Substrate-dependent NADPH oxidation activity was assayed in the presence of sodium laurate. To a sample of P450 BM3 (1 mL), sodium laurate (500 µL) was added. The reaction was initiated by the addition of NADPH and the reaction followed by observing the decrease in A_{340} measurements

4.5 Results

4.5.1 Growth of *B. megaterium* on complex and casamino acids

Growth on complex media was vigorous giving dense cultures after only 24 hr. Wet weight (wwt) and dry weight (dwt) analysis showed that 13 g/L of nutrient yields a biomass of 8 to 9 g/L (wwt) and a wwt/dwt of ~23 %. When *B. megaterium* colonies from complex solid media were transferred to casamino acid salts liquid media no growth was detected after an incubation period of 7 days. Cells of *B. megaterium* experienced an environmental shock when the change in nutrient base occurred and did not grow because of the partial shut down of these pathways. To over come this a growth stage on casamino acid salts solid media was incorporated. Samples of *B. megaterium* cultured on plates of complex media were sub-cultured onto fresh of casamino acid solid media in an attempt to switch on the pathways that were thought to be redundant in the colonies derived from complex media. To supply this nutrient several sources and varieties of casein were tested **(Table 4-2).** The biomass yields from the sources of casamino acid were undetectable and

no improvement was seen. In order to obtain suitable quantities of biomass for the induction and purification of P450 BM3 the use of semi-defined media was abandoned.

4.5.2 Induction of P450 material

The effect of pentabarbital on the biomass of *B. megaterium* was determined for the addition of inducer at a time corresponding to the mid-log growth phase. Pentabarbital was added at mid-log because the cells are in their most active growth phase and most able to adapt to the addition of a carcinogen. Growth ceased with the addition of 6 mM pentabarbital. Lower concentrations resulted in higher biomass production. A concentration of 5 mM was found to reduce the growth but does not induce cell death (by light microscopy) therefore this concentration was used in the induction phase.

4.5.3 Spectrophotometric assay of wash buffers and elution buffers

Cells from a large-scale fermentation (2 L) were induced for the production of cytochrome P450 by the addition of pentabarbital at mid log growth phase. By the analysis of A_{280} and A_{260} spectral data, protein and DNA were present in the homogenate and in the samples from both wash and elution buffers. However an attempt to record A_{450} spectral data (reduced dithionite spectra) showed that there was no cytochrome P450 enzyme present. Cell free extract in fractions from wash buffers were tested for the presence of a A_{450} absorbance maximum after the addition of sodium dithionite and bubbling CO for 1 min to indicate the presence of P450 material.

4.6 Conclusions

On the basis of the spectral data, no cytochrome P450 was present in the samples tested. This contrasts with the findings **(Black *et al.*, 1994)** who showed that the catalytic quantities were present using the above method. Catalytic assays for the activity of P450 BM3 confirmed the results of the spectral data.

Since the developments made, yielded no induction of P450 BM3 in the complex media it is concluded that the presence of P450 BM3 is transitory, expressed in low quantities, or not induced using this method.

The lack of P450 BM3 may possibly be because of the different feeding strategies followed in this section and to that documented **(Black *et al.*, 1994).** Media, rich in nutrients may have resulted in colonies susceptible to changes in the environment, such as the change to a week media or the presence of toxins, pentabarbital. The use of *B. megaterium* to produce the soluble form of P450 was abandoned. At this stage, the three component soluble P450cam from *Pseudomonas putida* or a high expression source of P450 BM3 was investigated.

4.6.1 Search of a high expression source of P450 BM3

P450 BM3 is a single polypeptide chain of amino acids. The cloning of P450 BM3 into a suitable host was found to be achieved **(Miles *et al.*, 1992).** In the next section the production of *E. coli* and purified P450 BM3 is investigated. This route was chosen over the P450cam because of the apparent ease of growth induction and isolation **(Black *et al.*, 1994).**

It was thought that some biosynthetic pathways in the colonies *of B. megaterium* cells grown on complex media were not induced. These pathways were thought to be essential for the growth on the semi-defined casamino acids salts media.

No improvement in the growth characteristics was seen and so the casamino acid source was thought to be deficient in a key amino acid (auxotropic) nutrient.

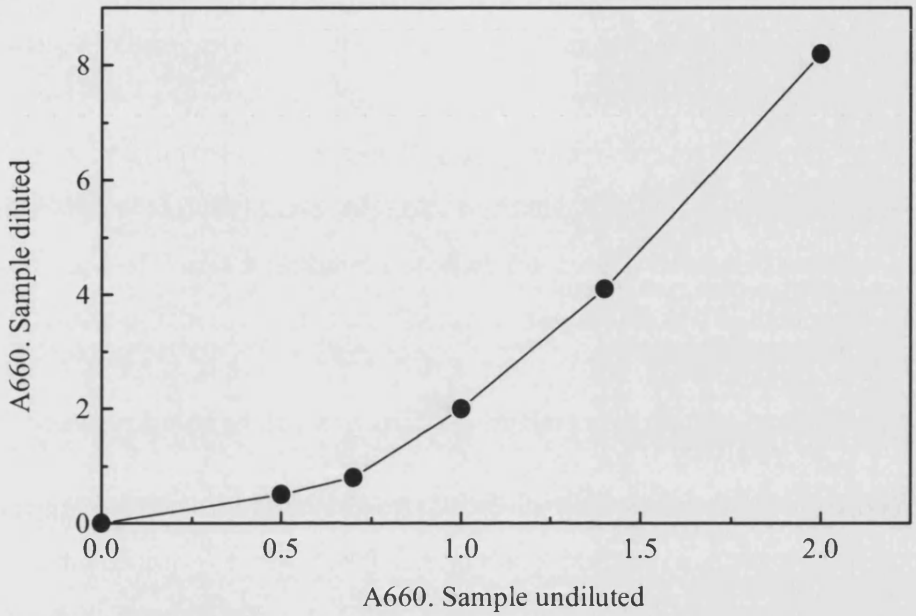


Figure 4-2 The absorbance of *B. megaterium* at 670 nm.

Absorbance measurements made for diluted and undiluted sample. Graph shows that at absorbance values above 0.8 the function becomes increasingly non-linear and that A_{670} values above this value diverge from the actual value.

5. Isolation of P450 BM3 from *E. coli*

5.1 Introduction

Native *B. megaterium* has been found to be a poor source of P450 BM3 **(Section 4).** The gene *CYP102* encoding cytochrome P450 BM3 has been cloned in *E. coli* **(Miles *et al.*, 1992).** P450 BM3 has been over expressed and purified to homogeneity **(Miles *et al.*, 1992).** In this section colonies of P450 BM3 were obtained and quantities of high purity for solvent inhibition studies were made **(Dellar & Turner, 1997[1]).** The purification was based on a modified method **(Munro *et al.*, 1994).**

5.2 Abstract

B. megaterium ATCC 14581 was grown on LB broth with ampicillin **(Black *et al.*, 1994)** to a scale of 100 mL. Cytochrome P450 BM3 production was induced by the addition of IPTG. High cell densities of *E. coli* were obtained. Spectrophotometry and light microscopy were used to record the biomass. Cells from complex media were harvested, ruptured and an attempt made to purify P450 BM3 by affinity chromatography. P450 BM3 was detected using spectrophotometric and catalytic techniques.

5.3 Materials

5.3.1 Source of *E. coli*

Stocks of transformed *E. coli* XL-1 Blue (pJM25) were obtained from Dr. A.W. Munro (University of Edinburgh) as colonies on solid (LB.ampicillin) media.

5.3.2 Solid media

13 g/L LB Agar, Miller (Sigma chemical Co., UK), 50 μg/mL ampicillin in RO water was autoclaved (121 °C, 20min.) and 30 mL of the autoclaved solution poured into each petri

dishes. Solid media was left to cool and incubated at 30 °C for 5 days. Contaminated plates were discarded.

5.3.3 Liquid media

24 g/L LB broth, Miller, (Sigma), 50 μg/mL ampicillin was autoclaved (121 °C, 20 min).

5.3.4 Chromatography

Ion Exchange Chromatography was achieved using fast protein liquid chromatography (FPLC) apparatus (Pharmacia Biotech, Sweden). A P-1 variable speed pump was used to load and elute the protein sample from the DEAE Sephadex A-25 cation exchange matrix. The samples were collected by a fraction collector (Pharmacia FRAC 100).

5.3.5 Affinity Chromatography

2',5' ADP sepharose (Sigma, UK) was used in the affinity separation of P450 BM3.

5.3.6 Gel filtration Chromatography

Size exclusion chromatography was used in the final step to concentrate the sample of P450 BM3.

5.3.7 Spectrophotometry

UV spectra, spectrophotometric assays and absorbance readings were recorded (ATI Unicam UV2 UV/vis photospectrometer). A water bath and immersion thermostat (VFK provided Grant, UK) temperature regulation.

5.3.8 Light microscopy

A light microscope (Olympus CH) was used for all microscopy work. Growing microorganisms were viewed (D Plan 100 oil optic) with an overall magnification of 1000x. Cedar wood oil was used to lubricate the lens and the cover plate.

5.3.9 Buffers

All buffers used are summarised bellow **(Table 5-1).**

Buffer	Buffer Components
A	50 mM Tris, pH 7.25
B	10 mM potassium phosphate, pH 7.7
C	100 mM potassium phosphate, pH 7.7
D	200 mM potassium phosphate, 2'5'-AMP, pH 7.7
all buffers	2 mM β-mecaptoethanol, 1 mM leupeptin, 1 mM EDTA, 1 mM PMSF,

Table 5-1 Buffers used in the harvesting of *E. coli* and the down stream processing of P450 BM3

5.4 Methods

5.4.1 Solid media and maintenance of *E. coli*

E. coli, DH5a/pBM25 was obtained as a one week old culture on LB.ampilcillin agar. Glycerol stocks of colonies were added to 20% (w/v) glycerol and stored at -70 °C. Fresh cultures of *E. coli* were taken from frozen stocks and grown on solid LB agar with 50 μ g/mL ampicillin.

5.4.2 Shake flask fermentation

Cultures (5 mL) were produced from a colony of *E. coli* from a solid media culture incubated for 4 days at room temperature. A line of *E. coli* was required to inoculate LB.ampicillin broth (100 mL) in a 500 mL baffled shake flask. Cells of *E. coli* were then incubated (37°C, 200RPM). The production of P450 BM3 was induced by the addition of IPTG when an A_{670} was between 0.3 and 0.7. After the addition of ampicillin the incubation temperature was reduced to 30 °C.

5.4.3 Induction of P450 BM3

The *E. coli* clone obtained (DH5a/pBM25) contained the IPTG inducible *lac* promoter. P450 BM3 production was promoted by the addition of IPTG to a final concentration of 500 μg/mL.

5.4.4 Cell Harvest

Cells of *E. coli* were harvested for 15 hr after the addition of ampicillin. The cells were centrifuged at 10,000*g* (JA2 MI Beckman, UK) for 20 min for 4 °C. This was adequate for producing a clear cell free supernatant. The cell pellet from centrifugation was suspended in buffer A **(Table 5-1)** and centrifugated (10,000*g*) to produce clean cell pellet free from growth media. Cells were stored at -20 °C until further use.

5.4.5 Cell rupture by freeze thaw cycle and sonication

The use of homogenisation as in the previous section caused a high concentration of fragmented DNA to be released into the supernatant. This material proved difficult to remove and so the use of an alternative method of cell rupture that did not cause DNA fragmenting was found. The effect of freezing and thawing followed by sonication was tested. Compacted cells suspended in buffer A **(Table 5-1)**, at 4 °C. They were frozen and thawed twice to disrupt the cell membrane, and were then sonicated at 4 °C for 10 min.

5.4.6 Cell free extract

Cell debri was removed from the protein extract by centrifugation (10,000g, 20 min. 4°C, Beckman J2MI).

5.4.7 Ammonium sulphate precipitation

Ammonium sulphate [$(NH_4)_2SO_4$] precipitation was used to produce a fractional precipitation of cell proteins. Ammonium sulphate was chosen because it; causes the precipitation of most proteins and it does not have a large heat of solution, so that the heat generated is low and easily dissipated. Even a 4.04 M saturated solution (20 °C) has a low

density (1.24 g/cm^3) therefore it does not interfere with the sedimentation of most precipitated proteins by centrifugation and its concentrated solutions prevent or limit most bacterial growth and in solution it protects many proteins from denaturation.

The volume of protein was determined and the solution placed on ice. Solid ammonium sulphate was then added (with stirring over a period of 2 min) to a final solution concentration of 30% (w/v). The use of a magnetic plate and flea was found to be necessary to reduce concentration gradients of ammonium sulphate within the vessel during the addition. After 20 min. the supernatant was removed from the precipitate by centrifugation (10,000*g*, 20 min. 4 °C) and poured into a ice cold beaker. Further ammonium sulphate was added to a final concentration of 60% (w/v). After 20 min. the solution was discarded and the pellet corresponding to the 30% to 60% (w/v) ammonium sulphate fraction was recovered. Solid fractions were stored at -20 °C until use.

5.4.8 Dialysis

Ammonium sulphate pellets were suspended in a small volume of buffer A **(Table 4-1).** To reduce the ionic strength of the protein solution, dialysis against buffer A **(Table 4-1)** was required. Space in the dialysis tubing (12,000 RMM cut off, BDH, UK) was required to allow for the increase in volume inside the tubing. Insoluble material was removed from the sample by centrifugation (20,000*g*, 20 min., 4 °C).

5.4.9 Ion Exchange Chromatography

An Ion Exchange Column (DEAE Sephacel, 100 mL, Sigma) was equilibrated with buffer A (200 mL) **(Table 5-1).** The supernatant produced by dialysis was then loaded onto the column manually. The non-binding proteins were then removed from the column by the addition of buffer A (200 mL) **(Table 5-1)** at a rate of 1 mL/min. To elute bound proteins, a 500 mL gradient of KCl (0-500 mM) was used in buffer A **(Table 5-1).** Fractions were collected and the A_{450} recorded **(Figure 5-1).**

5.4.10 Ultrafiltration

Peak fractions (dark red colour) obtained by Ion Exchange chromatography were concentrated using ultrafiltration. Centriprep microseparator with 30,000 RMM cut off (Amicon, UK) were used.

5.4.11 Affinity Chromatography

A 2',5' ADP sepharose column matrix (Sigma, UK) was used in the affinity separation of P450 BM3. 40 mL of column matrix contained in a FPLC column (HR 10/30, [1 x 20 cm], Pharmacia Biotech, Sweden). Buffer B (100 mL) **(Table 5-1)** was used to equilibrate the column. Concentrated protein was then added to the column at a rate of 2mL/min. and the column washed with of buffer B (100 mL) **(Table 5-1)**. Buffer C (40 mL) **(Table 5-1)** was used to remove material with an ionic interaction to the matrix. Protein was eluted from the column by the addition of buffer D (80 mL) **(Table 5-1).**

5.4.12 Spectrophotometry assay of P450 material

The yield of each step of the purification process was recorded by measurements of the Soret band of the sample at A_{418} using $\varepsilon = 143/mM/cm$ **(Figure 5-1). (Black *et al.*, 1994).**

5.4.13 Measurement of the P450 concentration

The concentration of cytochrome P450 was determined as described in the literature **(Narhi & Fulco, 1987)**. Reduced spectra of the sample were obtained by the addition of 2-3mg of solid sodium dithionite ($Na_2S_2O_4$) **(Figure 5-1).** A mixture of carbon monoxide (CO), and nitrogen (N_2), was gently bubbled through a sample of dithionite reduced protein solution for 1 min and the spectra recorded **(Figure 5-1).** This was sufficient to saturate the sample with gas. Difference spectra were obtained by the difference in the spectra of the dithionite reduced and reduced CO bound spectra.

The concentration of P450 protein could be determined from the absorbance at A_{450} from the difference spectra using $\varepsilon = 91/mM/cm$ **(Narhi & Fulco, 1992)**.

5.4.14 Substrate binding

The effect of fatty acid substrate on the spectrum of the oxidised form of P450 BM3 was tested. The spectra of purified P450 BM3, was obtained in buffer A. Sodium laurate was added to a final concentration of 500 μM and the spectra repeated. A difference spectra was obtained by the difference of the two spectra **(Figure 5-2).**

5.4.15 Catalytic assay for P450 BM3

5.4.15.1 Reduction of NADPH

The reduction of NADPH was monitored in a 1 cm glass cuvette at 340 nm. A 40 nM solution of purified P450 BM3 was added to 200μM NADPH, in 100 mM potassium phosphate pH 7.7. Sodium laurate (100 mL) was then added to a final concentration of 250 μM and a final volume of 1.5 mL. The rate of reaction was monitored spectrophotometrically at A_{340}.

5.4.15.2 Gas chromatography (GC)

GC was used to determine the rate of substrate (sodium laurate) loss and the rate of the production of the hydroxy product.

5.5 Results

5.5.1 Growth of *E. coli* on LB.ampicillin

Good growth was observed using the LB.ampicillin media. After 5 days on solid media, large colonies were formed. On liquid media exponential growth phase occurred within 5 hr. Stationary growth phase was observed at 48 hr. Cells of *E. coli* were harvested before the onset of stationary phase as this was thought to result in a decrease in P450 BM3 yield. The antibiotic ampicillin added to the media prevented growth of opportunistic organisms. These may have infected the culture and compete with the ampicillin resistant *E. coli* used. Expression of P450 BM3 decreased rapidly from cultures stored at 4 °C. For this reason the technique of sub-culturing was not used. Cultures of

E. coli were taken from 20% (w/v) glycerol stocks stored at -70 °C. Reduction in expression was attributed to plasmid loss.

5.5.2 Storage of purified P450 BM3

When the concentration of P450 BM3 in the sample was recorded, the solution was stored in sterile glycerol (20% (w/v))

5.5.3 Spectra

Production of cytochrome P450 was induced by the addition of IPTG to a final concentration of 500 μg/mL. The spectrum of the oxidised form of the protein was first recorded, the sample treated with dithionite, and the spectrum of the reduced protein was taken. Finally, the reduced protein was bubbled with CO for 1 min and the reduced CO bound spectrum was recorded.

The spectra of the sample oxidised with the addition of IPTG, has absorbance maxima at 390 and 420 nm **(Figure 5-1)**. This is a characteristic 'low spin' spectrum for a P450 Cytochrome. The spectra of the sample reduced with dithionite, has a single absorbance maxima at 410 nm **(Figure 5-1)**. This is a characteristic 'high-spin' spectrum for a P450 Cytochrome. The spectrum of the sample first reduced with dithionite, then bubbled with CO has a strong absorbance at 450 nm **(Figure 5-1)**. This is characteristic of the reduced CO spectrum of a purified sample of a cytochrome P450.
The three spectra **(Figure 5-1)** indicate that the sample is has a high concentration of Cytochrome P450.

The spectra of purified P450 BM3, was obtained in buffer A. Sodium laurate was added to a final concentration of 500 μM and the spectra obtained. The 'difference spectra' was obtained by the plotting numerical difference of the absorbance at each wavelength the two spectra **(Figure 5-2)**.

5.5.4 Spectral characteristics

The oxidised, reduced and the reduced CO bound spectra of this protein has absorption maxima corresponding to other P450 enzymes but in particular correspond exactly to cytochrome P450 BM3 **(Narhi & Fulco, 1992)**. The oxidised form has an absorbance peak at 418 nm and a shoulder at 394 nm. This corresponds to iron in the ferric (Fe^{III}) form in the heme. A_{418} corresponds to low spin (hexacoordinate) form of iron and A_{394} corresponds to iron in the high spin (pentacoordinate) form. Both peaks can be seen in the oxidised form indicating that the heme is only partially oxidised. The reduced form of P450 BM3 had an absorbance profile maxima at A_{418}.

5.6 Conclusions

The production and isolation of P450 BM3 from *E. coli* cloned for its expression was a superior method when compared to the natural organism *B. megaterium.* P450 BM3 was isolated and purified to high purity when determined spectrophotometrically.

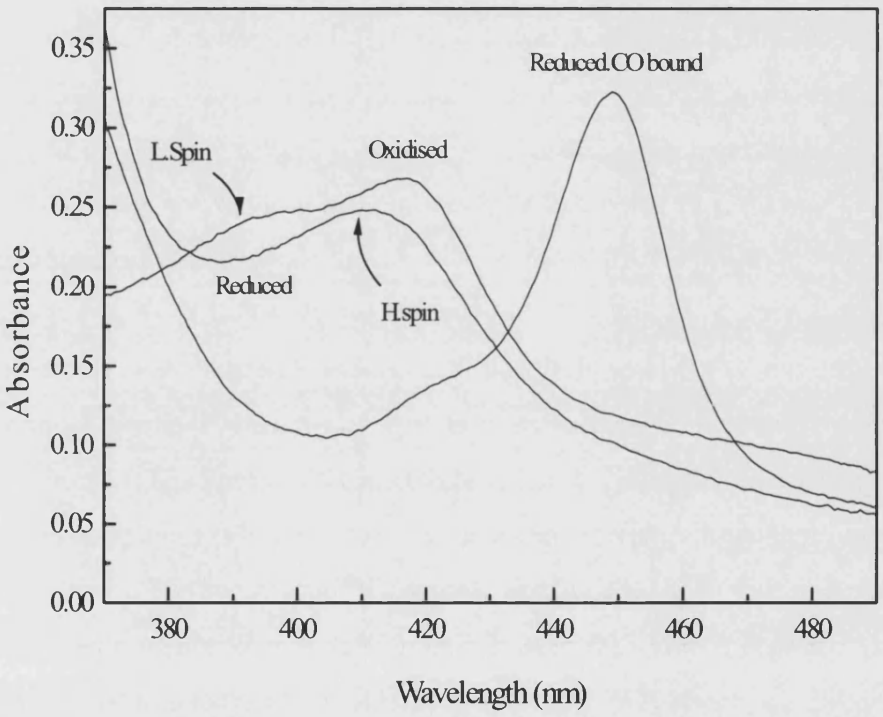


Figure 5-1 The spectra of purified cytochrome P450 BM3 from transformed *E. coli* DH5a/pBM25.

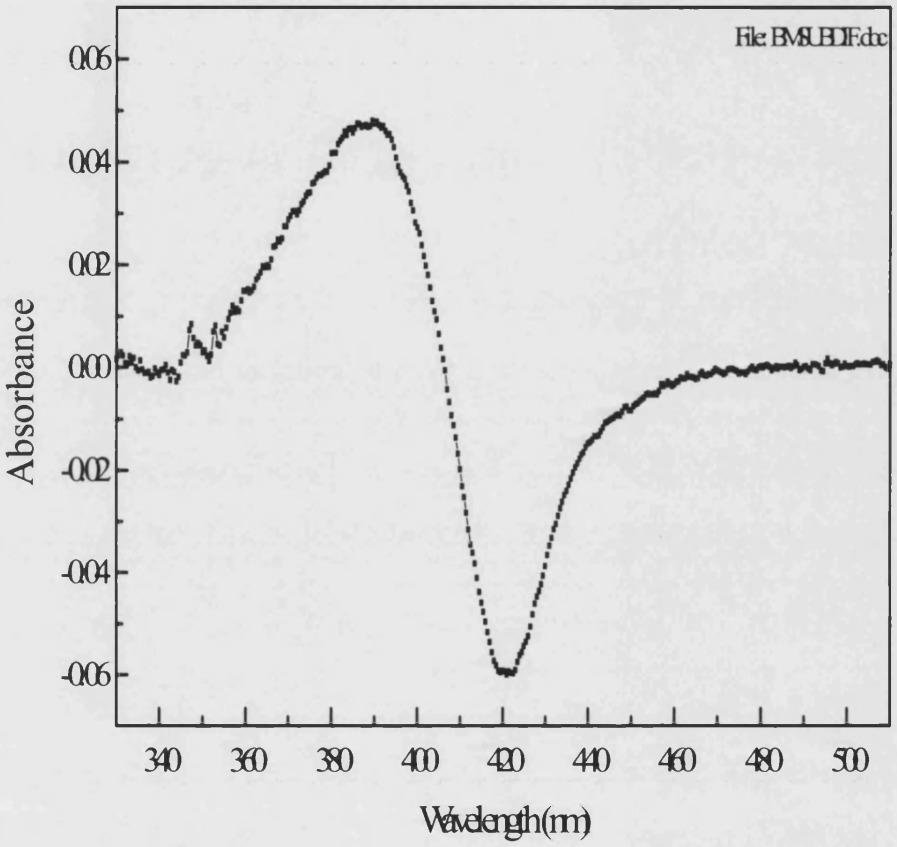


Figure 5-2 Effect of fatty acid substrate on the spectrum of the oxidised form of P450 BM3 (the difference spectra of purified P450 BM3

6. Effect of *n*-alcohols on the activity of cytochrome P450 BM3

6.1 Abstract

A series of primary alcohols from methanol to hexanol inhibits the activity of the catalytically self-sufficient cytochrome P450 BM3. The rate of inhibition of the hydroxylation of sodium dodecanoate increases with increasing potency as the molecular weight of the *n*-alcholol increases. The ability of *n*-alcohols to reduce the activity by 50% is limited to the first five members (C1-C5) of the group. This is due to the decreasing solubility of the alcohols in water. The aqueous concentration of solvent required for inhibition is of the same order as that required to inhibit the membrane bound hydroxylase from *R. stolonifer.*

6.2 Introduction

The effect of *n*-alcohols on the purified soluble P450 BM3 from *B. megaterium* can yield information about the direct effect of solvents on a P450 protein with similar structure to membrane bound P450 proteins.

B. megaterium has been identified as a source of soluble P450 **(Section 1).** *E. coli* transformed for the production of P450 BM3 has been cultivated in liquid media to produce high amounts of the enzyme P450 BM3 **(Section 5).** P450 BM3 has been purified to a concentration of 114 μM, 13.6g/L determined from the Soret band of the sample at $A_{450\text{-}490}$, using ε = 91/mM/cm **(Black *et al.*, 1994).** This enzyme will now be used in the hydroxylation of lauric acid. The effect of *n*-alcohols on the initial rate of the hydroxylation will be investigated. Earlier work suggests that the membrane bound P450 enzyme (P45011α) in *R. stolonifer* is inhibited when the concentration of a series of primary alcohols reaches a critical molar concentration in the membranes. In this work we have tested the effect of these alcohols on the activity of the soluble hydroxylase cytochrome P450 BM3 which is naturally present in *B. megaterium.* P450 BM3 has a

similar structure as the one described in *R. stolonifer* but it is not membrane associated. It can therefore be used to determine the direct effects of the alcohols on a cytochrome P450 without any complicating effects arising form the membranes.

6.3 Materials

Methanol, ethanol, propanol, *n*-butanol, *n*-pentanol and *n*-hexanol analytical grade (Sigma Chemical Company, UK). β-NADPH was obtained as the tetra-sodium salt (Sigma, UK). Sodium laurate (Sigma, UK) was stored as a 2 mM stock solution, pH 7.8 at 4°C.

6.3.1 Protein handling

Cytochrome P450 BM3 was prepared as a concentrated solution in a 100 mM Phosphate buffer, with glycerol 20% (v/v) pH 7.5 **(Section 5).** The enzyme was stored at -20°C until used at a concentration of 11.4 nM.

6.3.2 Spectroscopy

UV spectra, spectrophotometric assays and absorbance readings were performed (ATI Unicam UV2 -100 spectrometer) using standard 1 mL quartz glass cuvettes (Hellma®, UK). NADPH-dependant fatty acid hydroxylation was measured at 37 °C in 100 mM phosphate buffer containing 200 μM sodium laurate and 100 μM NADPH (ε_{340}, 6.2 mM^{-1} cm^{-1}).

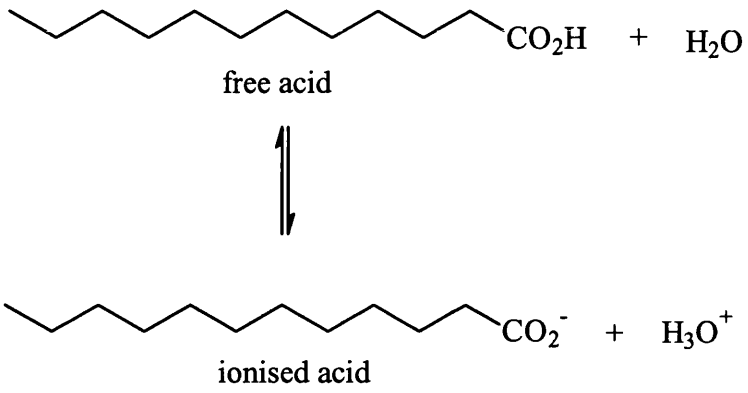
When alcohols were added to acrylic cuvettes, the surface of the cuvettes turned opaque. Quartz cuvettes were to be resistant to alcohols and were used in the spectrophotometric measurements.

6.3.3 Assay development

6.3.3.1 Lauric acid

Lauric acid was found to have a solubility of 300 μM when dissolved in water to yield a solution of pH 4.0. The sodium salt is soluble to 2 mM yielding a solution of pH 7.0. The increased solubility of sodium laurate reflects the increased tendency of Na^+ ions to

dissociate relative to H^+ ions. The solubility of lauric acid increases with the pH of solution since this shifts the equilibrium **(Equation 6-1)** to the production of the ionised acid. This equilibrium has been found to be slow therefore great care was taken in producing solutions of fatty acids.



Equation 6-1 The two states of long chain fatty acids

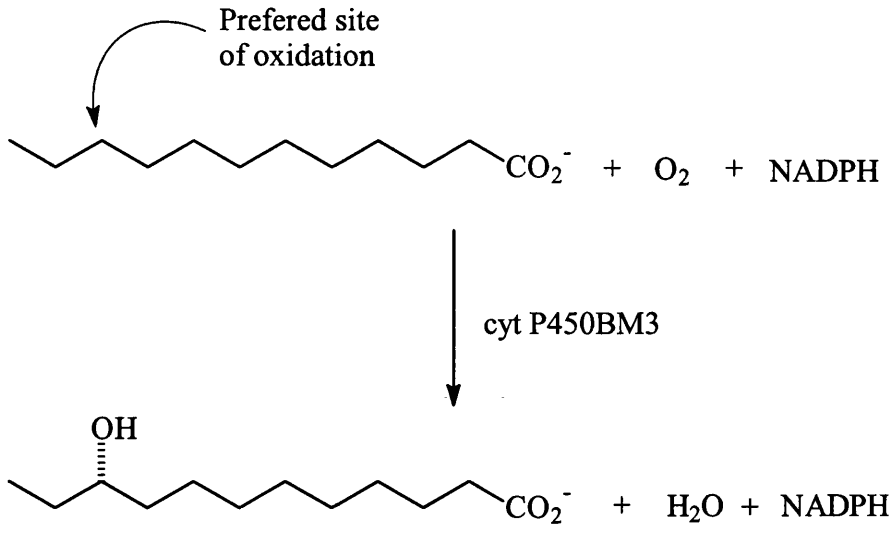
The presence of ions in solution decreases the solubility of sodium laurate therefore a stock solution of 2 mM in water was used.

6.3.3.2 NADPH

NADPH is only stable when in a dry form. Stability increases as temperature decreases and -70 °C was found to stabilise the reduced form.
Rate of sodium laurate hydroxylation in the absence of NADPH is negligibly slow compared to the rate of reaction in the presence of NADPH **(Equation 6-2).**

6.3.3.3 Kinetics of the reaction

The concentrations of substrates were chosen such that the loss of substrate had no measurable effect on the activity over the range of measurement. The effect of lauric acid concentration on the initial rate was also tested, and initial activities were found to exhibit classic Michaelis-Menten kinetics. The K_M value for the hydroxylation of lauric acid under the conditions chosen was about 73 μM **(Figure 6-1).**



Equation 6-2 The (ω-2) hydroxylation of sodium laurate

6.3.3.4 Effect of pH on the activity

The effect of pH was investigated with the addition of strong acid (HCL) and strong base (NaOH). The activity of P450 BM3 was found to describe the classic bell shaped curve. The maximum hydroxylase activity was at pH 7.5 **(Figure 6-2).**

6.3.3.5 Ionic strength

The effect of ionic strength was tested using a range of of potassium phosphate concentrations in the range up to 200 mM. The hydroxylase activity increased with the ionic strength, most activity was recorded at a concentration of 100 mM. At higher concentrations of phosphate, the hydroxylase activity decreased **(Figure 6-3).**

The non-specific depletion of NADPH catalysed by water in 100 mM phosphate buffer P450 BM3 and alcohol was shown to be undetectable at (A_{340}). Ester formation from the reaction of lauric acid and alcohol was not investigated and it is thought to be unlikely at neutral pH.

6.3.3.6 Oxidation of alcohols

Alcohols may oxidise NADPH, effectively removing NADPH from the reaction mixture. Since we are measuring the reduction of NADPH to determine the rate of hydroxylation the concentration of NADPH was measured in the presence of alcohol without sodium laurate. At the concentrations used in the hydroxylation experiments no reduction of NADPH was detected.

At the experimental concentrations there was no detectable de-coupled oxidation of (alcohol oxidation) NADPH, was measured in the absence of sodium laurate.

Ascending the homologous series of alcohols the solubility decreases **(Table 6-1).** As the chain length increased the concentration of alcohol required to inhibit P450 BM3 quickly rose to that of the same order of the maximum solubility in water. A saturated solution of pentanol was used to inhibit the reaction by 50%. Saturated solutions were obtained by mixing an excess of alcohol with water to produce a liquid two-phase system. A syringe (Hamilton, UK) was used to remove a sample of alcohol in water. This solution was tested for its ability to inhibit the hydroxylation activity at fixed time intervals after mixing. It was found that the partitioning was equilibrated after 2 min of mixing for pentanol.

***n*-alcohol**	**Density (g/mL)**	**Poct**	**Maximum aqueous solubility (M)**
Methanol	0.7914	-0.76	32
Ethanol	0.7893	-0.24	10
Propanol	0.8035	0.28	3.2
Butanol	0.8098	0.80	1.0
Pentanol	0.8144	1.30	0.250
Hexanol	0.8136	1.8	0.0501

Table 6-1 Solubility of *n*-alcohols in water at 25 °C **(Bell, 1972).**

6.3.4 mechanics of the hydroxylation assay

Sodium laurate was prepared as a 2 mM stock solution in water and used within one day. NADPH was prepared from dry stock at -70 °C to a concentration of 15 mM and kept on ice. It was found that the activity of P450 BM3 decreased rapidly when stored at low concentrations. Therefore was important to keep the concentration of P450 BM3 high until required. P450 BM3 was used in the assay at a final concentration of 11.4 nM. P450 BM3 concentrate (114 μM) was removed from -20 °C storage, diluted to 1.14 μM to produce stock solutions that was stored on ice and used within one day.

It was found that the injection of concentrated P450 BM3 to activate hydroxylation gave unreliable kinetics because of the high viscosity of the glycerol in the enzyme solution. Therefore 10 μL of the stock solution (1.14 μM) was diluted to a final volume of 100 μL, in 100 mM phosphate buffer pH 7.5 to reduce the viscosity of the solution immediately before use.

It was also important to inject P450 BM3 in the same concentration of buffer as in the cuvette, since this promoted fast mixing and reproducible kinetics. The rate of hydroxylation of sodium laurate in the absence of alcohol over a period of 15 s was taken as 100% activity. Various quantities of alcohols were then added to the system and the effect on the initial activity recorded.

6.3.4.1 Standard procedure

1 M phosphate buffer (100 μL), RO water (1090 μL), 2 mM sodium laurate and 15 mM NADPH, (10 μL), was added to a 1.5 mL quartz cuvette and the initial A_{340} value recorded. Hydroxylation was then initiated by the addition of of 114 nM enzyme (100 μL) and the A_{340} recorded.

6.4 Results and Discussion

All the alcohols tested, inhibited the hydroxylation of sodium laurate **(Figure 6-4).** Their potency as inhibitors increased with their molecular weight **(Figure 6-6).** Pentanol shows a 50 % inhibition at about 0.1x the molar concentration at which methanol has a similar effect (63 mM and 0.7 M respectively) **(Figure 6-6).**

A saturated aqueous solution of hexanol inhibits the hydroxylation by about 25 %. A larger degree of inhibition is prevented because the hexanol to too insoluble to reach a sufficient concentration, of about 55 mM in the aqueous phase. Nevertheless it is clear from the data **(Figure 6-6)** that it is a more effective inhibitor than pentanol since there is a detectable loss of activity even at a concentration of 10 mM in the aqueous phase. None of the other alcohols inhibit the enzyme at this concentration.

The apparent K_m for sodium laurate hydroxylation increases to about 150 μM when the activity is measured in the presence of 0.7 M methanol, which is close to the IC_{50} value. There is a small decrease in V_{max}, but the data does not differ significantly for that expected of a competitive inhibitor where the K_i (for methanol) is about 0.5 M.

In the previous experiments with the membrane bound hydroxylase from *R. stolonifer*, the aqueous concentration of methanol and pentanol necessary to inhibit the activity by 50% were about 2 M and 25 mM respectively **(Munro *et al.* 1994).** Although these figures are of the same order as the IC_{50} values for their inhibition of cytochrome P450 BM3 they are sufficiently different to warrant a more careful study of the former with techniques which measure the membrane concentrations of the solvents.

This can be achieved using whole cells of *R. stolonifer* and the methods developed in **Section 2.**

The concentration of alcohol required to inhibit hydroxylation by 50% (IC_{50}) was recorded for all alcohols. The average of three experiments is shown **(Table 6-2).**

n-alcohol	$Log_{(10)}$ IC_{50}	IC_{50} (mM)	$Log_{(10)}$ solubility	Solubility (M)
Methanol	-0.15	708	32	32
Ethanol	-0.35	447	1	10
Propanol	-0.78	166	0.51	3.2
Butanol	-1.05	89.1	0.00	1.0
Pentanol	-1.20	63.1	-0.60	0.25
Hexanol	*-1.26*	*55.0*	-1.30	0.0501

Table 6-2 Comparison of IC_{50} concentrations of alcohols and maximum solubility of alcohols in water. Figures in italic are predicted values based on experimental results.

As the homologous series is ascended from C_1 to C_6 the values of IC_{50} decrease and the toxicity increases. Hexanol inhibits the hydroxylation but the concentration required for IC_{50} (approx. 55 mM) is above the maximum solubility in water. The presence of phosphate ions limited the solubility even further.

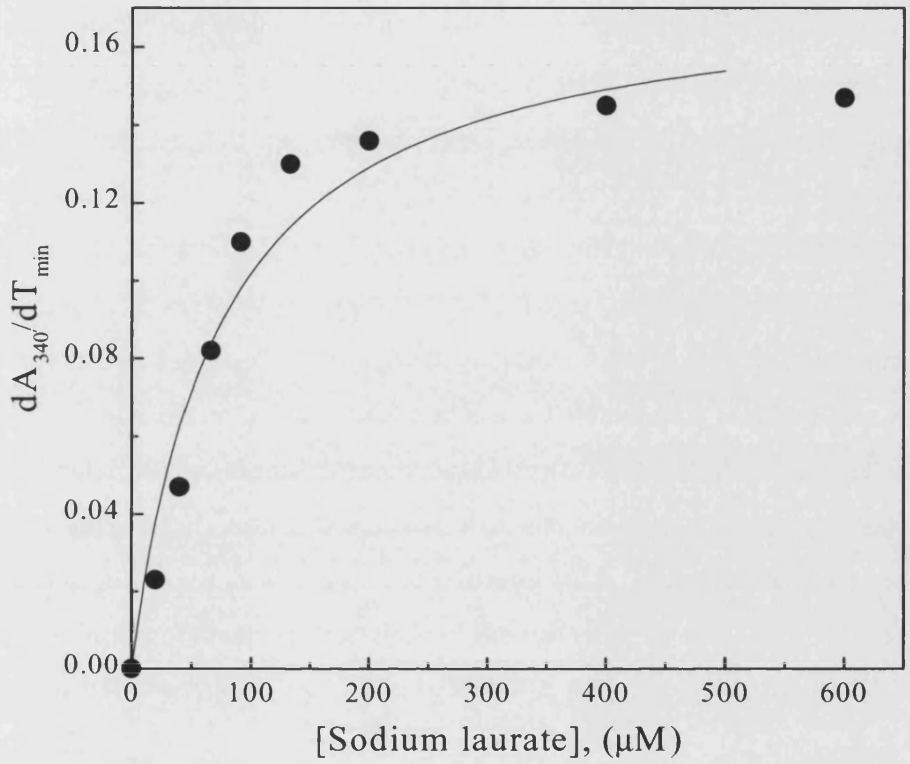


Figure 6-1 Effect of Sodium laurate concentration on the initial activity of P450 BM3.

Assay conditions: 11.4 nM Cytochrome P450 BM3, sodium laurate, in a 100 mM potassium phosphate solution, pH 7.5. Values of V_{MAX} (0.18 Units/min) and K_M (73 μM) were obtained using standard fitting function of a rectangular hyperbola with no weighing.

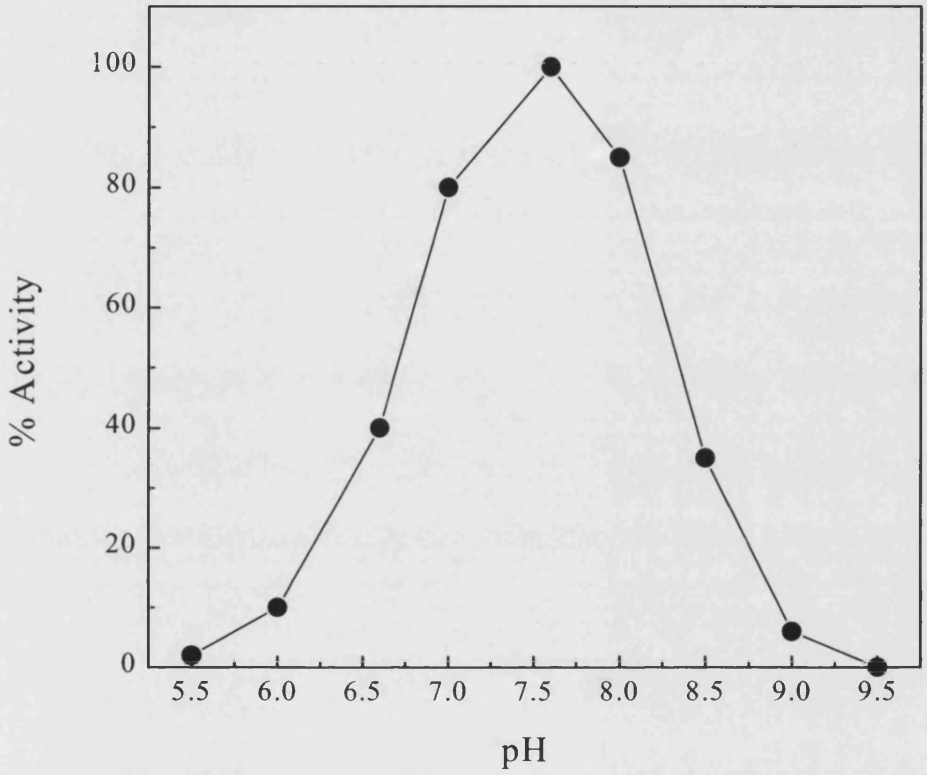


Figure 6-2 Effect of pH, on the initial activity of P450 BM3.

Assay conditions: 14 nM P450 BM3, 200 μM sodium laurate, in a 100 mM potassium phosphate solution.

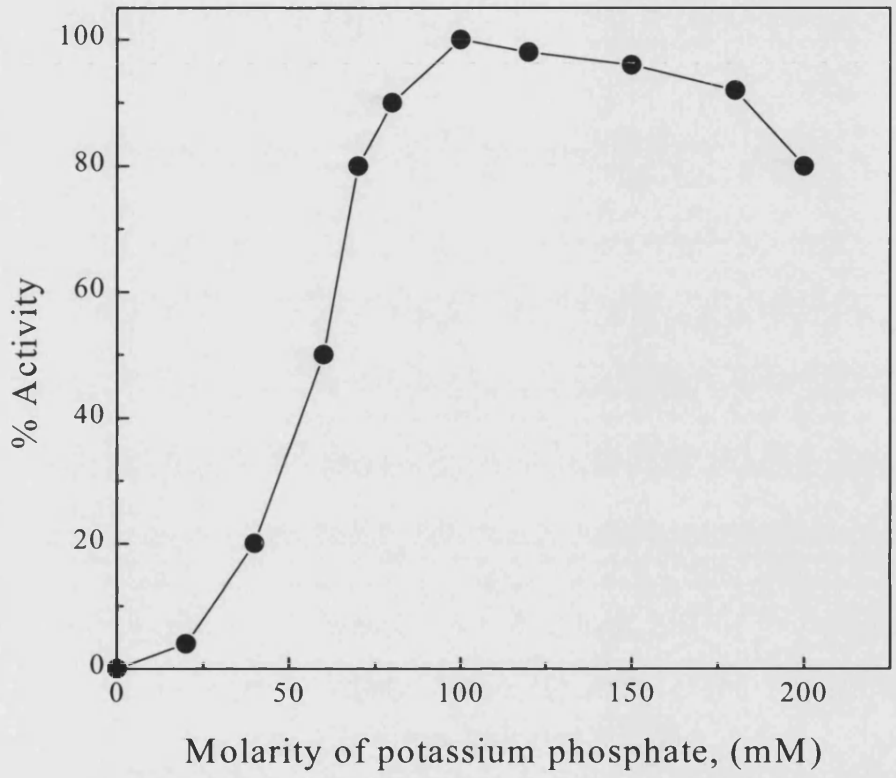


Figure 6-3 Effect of ionic strength of the solution on the initial activity of P450 BM3.

Assay conditions: 11.4 nM P450 BM3, 200 μM sodium laurate, in a potassium phosphate solution, pH 7.5.

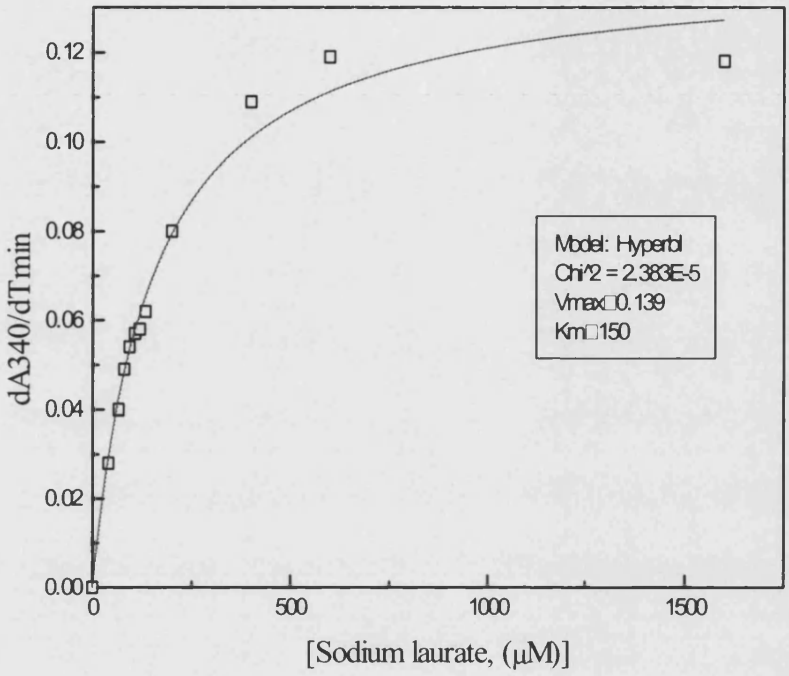


Figure 6-4 The effect of methanol on the initial activity of hydroxylation.
Assay conditions: as for Figure 6-1 with the addition of 700 mM of methanol.
This corresponds to IC_{50} with 100 mM sodium laurate.

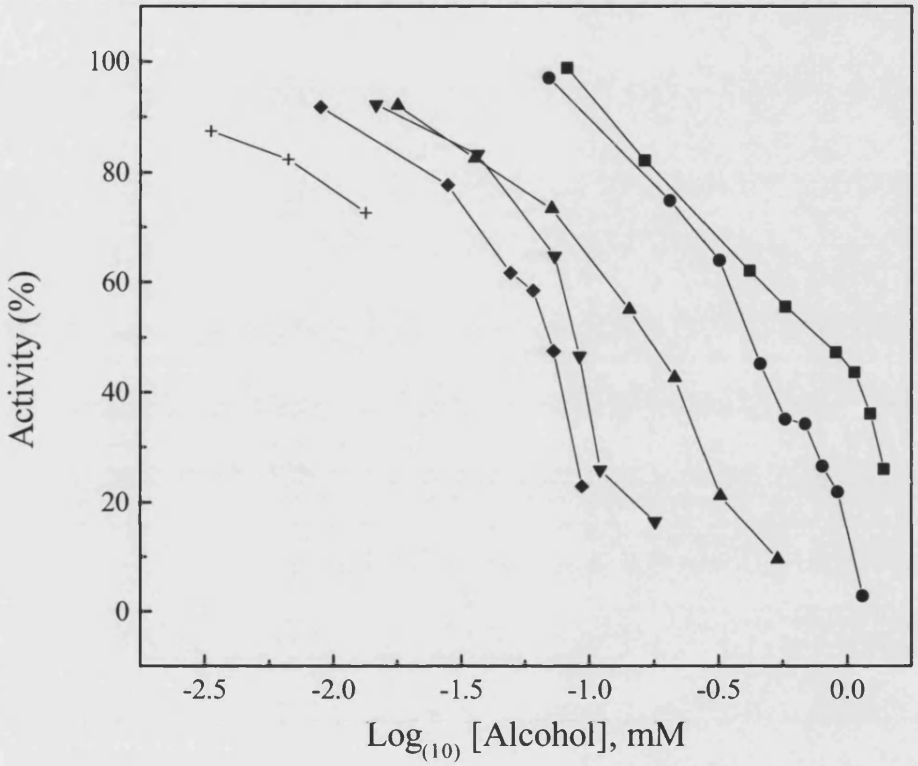


Figure 6-5 Effect of *n*-alcohols on the rate of sodium laurate hydroxylation. Assay conditions: 11.4 nM P450 BM3, 200 μM sodium laurate, and 100 μM NADPH in a solution of 100 mM potassium phosphate buffer, pH 7.5. Alcohols (■) methanol, (●) ethanol, (▲) propanol, (▼) butanol, (♦) pentanol, (+) hexanol.

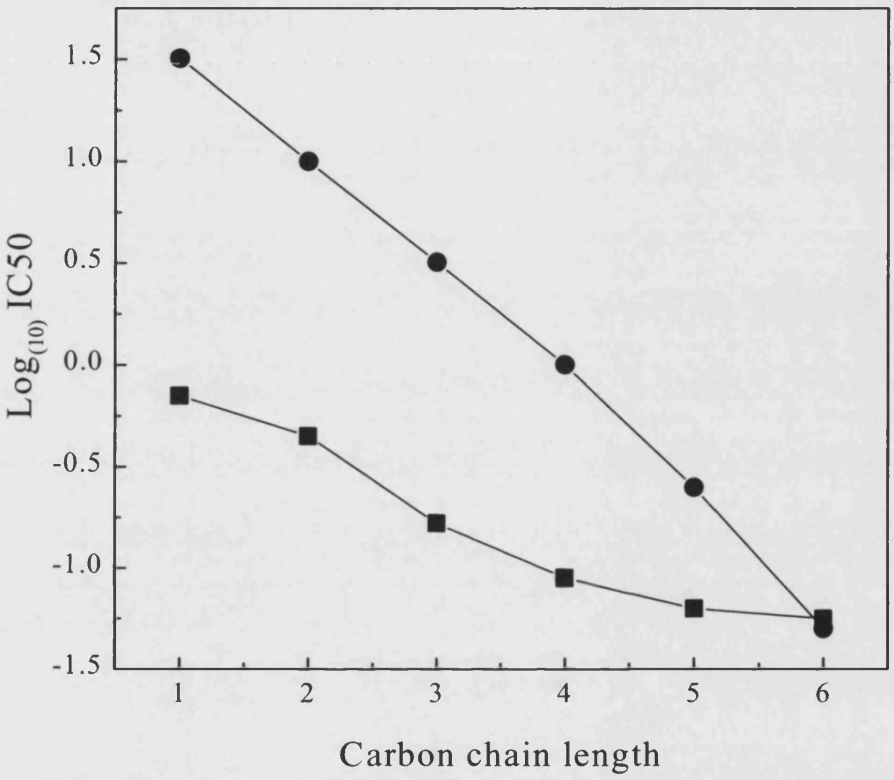


Figure 6-6 The inhibition of P450 BM3 with alcohols of increasing chain length.

(■) IC_{50} solubility of alcohols in water (●).

7. Hydroxylation of progesterone by whole cells of *Rhizopus stolonifer*

7.1 Abstract

(1) Whole cells of *R. stolonifer* were grown on solid and liquid potato dextrose media.

(2) The induction of P45011α with progesterone was determined at various times during the incubation.

(3) The production of P45011α was found to be independent of the time of induction.

(4) The effect of the time of induction on the final biomass on the culture was low at the concentration used to give good expression.

7.2 Introduction

The specific hydroxylation of progesterone to 11α-hydroxyprogesterone can be catalysed by the monooxygenase system P45011α in *R. stolonifer* **(Sonomoto, Nomura, Tanaka, et al., 1982).** The resolution of this hydroxylase into its three components and a subsequent reconstitution of the complete electron transports chain **(Breskvar, *et al.*, 1987),** showed that P45011α is a class I cytochrome P450 when characterised in terms of its redox partner **(Ravichandran *et al.*, 1993).**

It has been shown that P45011α is induced in *R. stolonifer* as a defence mechanism with hydroxy-progesterone excreted from the fungi **(Breskvar *et al.*, 1995).** The microsomal fraction of the mitochondrial membranes of *R. stolonifer* has been purified. Cytochrome P45011α has been reconstituted and shown to have simular activity as whole cells of *R. stolonifer* **(Breskvar *et al.*, 1987).** However the actual activity was measured by the reduction of NADPH and this contrasts with previous work **(Hanisch, 1978),** where no P45011α activity was found in microsomal preparations as determined by the transformation of progesterone to hydroxyprogesterone.

The effect of aqueous concentrations of *n*-alcohols on P45011α activity has been determined by the final concentration of hydroxyprogesterone over a 24 hr whole cell incubation period **(Osborne, 1990).** The activity of P45011α would be best observed during the initial reaction period, so that at a latter stage the effect of *n*-alcohols on the initial activity can be seen.

In these experiments whole cells of *R. stolonifer* will be grown to a suitable biomass and the induction of active P45011α investigated with the addition of progesterone. The rate of conversion of progesterone to 11α hydroxyprogesterone by whole cells of *R. stolonifer* will be followed by HPLC during the reaction **(Osborne, 1992).**

7.3 Materials

7.3.1 HPLC equipment

A Sperisorb Octyl 5 μm HPLC column was used for all steroid analysis (Sigma Chemical Co., Poole, UK). A C_8 reverse phase 1 cm guard column (Phase Separations) pre column was used to protect the main analytical column from damage and was changed after 2000 operations.

7.3.2 Buffers and Media

Buffer A: 50 mM potassium phosphate buffer pH 7.5 with 1.5 mM $MgCl_2$.
Potato dextrose agar (PDA), potato dextrose broth (PDB), progesterone and 11α-hydroxy-progesterone were obtained from Sigma Chemical Co., Poole, UK.
24 g/L PDA and 24 g/L PDB were used to cultivate *R. stolonifer* in solid and liquid phase respectively.

7.4 Methods

7.4.1 Organism

R. stolonifer ATCC 622b was obtained from Glaxo Welcome Group Research.

7.4.2 growth conditions

Cultures of *R. stolonifer* were obtained by the inoculation of PDA until heavy sporulation was noted. Sterile separation of spores from mycelia was achieved using a nylon mesh into a volume of water. Spore suspensions were counted using a heamocytometer counting chamber and centrifuged at 50,000 *g* for 1 hr (Beckman J2-MI ultracentrifuge). A known quantity of spores was used as inocula for 100 mL of PDB in 500 mL baffled flasks. Flasks and reaction vessels were all coated with (Sigma cote®, Sigma) before use. The pH of PDB was adjusted to 4.5 using phosphoric acid and steam sterilised (Denley BA852 Sovereign portable autoclave) at 121 °C for 20 min. Production of *R. stolonifer* was performed in an incubated shaker for 18 hr at 200 RPM, 28 °C.

7.4.3 Induction and harvesting

Progesterone was added to induce P45011α production at various times during the growth. The cells were harvested after 18 hr using a nylon mesh and washed with 1 L of buffer A **(Section 7.3)** until the eluent was clear.

7.4.4 Determination of biomass

Cells from 100 mL cultures were filtered through a Buchner funnel with Whatman filter paper for 3 min. The filtered cells were weighed (wwt) and oven dried at a temperature of 70 °C until constant weight (dwt). The dwt/wwt ratio was taken to be the ratio of the weight of cells before and after oven drying.

7.4.5 HPLC sample preparation

400 µL Samples of cell suspension were taken throughout the incubation and 50 mM hydrochloric acid (10 µL), was added to the samples to stop any reaction and precipitate cell debris. Acidified samples were spun for 1 min at 13,000 RPM. 10 µL aliquots of supernatant were then added to 990 µL of mobile phase **(Table 7-1).** These samples were analysed for progesterone and hydroxy-progesterone.

Component	Setting
Mobile phase	methanol/water (80:20 v/v)
Pressure	1000 p.s.i.
Flow rate	1.4 mL/min
loop volume	50 μL
Temperature	20 °C
Detection λ	254 nm
Separation time	4 min.

Table 7-1 HPLC conditions for the detection of progesterone and 11α-hydroxy-progesterone

7.4.6 Assay of P45011α activity

A spectrophotometric scan of progesterone and 11α-hydroxyprogesterone showed that the maximum absorbance occurs at 254 nm. 50 μL samples of reaction media, dissolved in to mobile phase (2 mL), were assayed using a reverse phase HPLC **(Table 7-1).**

7.5 Results

7.5.1 flask preparation

Osborne (1990) used flasks that had been coated with silicon to prevent fungi adhering to the vessel. This was found to be very important as the glassware that had not been treated with silicon proved to be a good anchor for growth, allowing the culture to be removed from solution

7.5.2 Inocula levels

Osborne (1990) reported two types of growth forms. Filamentous growth was obtained by inoculation in 1 L of culture medium with $5x10^8$ spores, $500x10^6$ spores/L. Pelleted growth however, occurred at an inoculum dose of $1.7x10^8$ spores in 0.75 L of culture

medium, $230x10^6$ spores/L. Although where spores inoculum ranged from $0.2x10^6$ to $60x10^6$ spores/L no pelletted growth was observed.

Low spore levels resulted in long and variable lag phases preventing growth in some cases **(Figure 7-1).**

Initial inocula of $15x10^6$ spores/L gave reproducible growth with no signs of a variable lag phase. One plate of mature culture can yield up to $3x10^6$ spores and so this lower inocula was useful since one 10 day incubation of *R. stolonifer* on PDA could give enough spores for one inoculation of 0.2 L of liquid culture. In contrast the method developed by Osborne requires $500x10^3$ sp./L. This corresponds to 12 petri dishes of *R. stolonifer* for 0.1 L of culture. Spores at a concentration of $15x10^6$ spores/L were used to inoculate cultures in all further work.

7.5.3 Effect of media concentration

The effect of substrate concentration on the growth characteristics was determined for PDB media. It was found that 24 g/L PDB produced a high biomass yield when compared to the concentration of PDB **(Figures 7-2 & 7-3).**

7.5.4 development of induction protocol

To induce the production of P45011α **Osborne (1993),** used 2 g/L progesterone (6.36 mM). However progesterone, is not soluble at this concentration in buffer A **(Section 5.3).** The effect of the induction time and amount of progesterone on the final biomass and the activity of P45011α were not documented in the thesis.

It was initially assumed that only progesterone in the aqueous phase would promote the induction of P45011α and act as a substrate. Thus the excess progesterone that existed in the solid phase would be unnecessary and therefore an investigation into the solubility of progesterone in a range of media was explored **(Table 7-2).** Aqueous progesterone levels were obtained by producing an excess solution of progesterone. A sample of the solution was removed, filtered and assayed for progesterone using HPLC **(Table 7-1).**

Media	Maximum solubility (g/L)
2 mM Potassium phosphate buffer	0.38
10 mM Potassium phosphate buffer	0.09
20 mM Potassium phosphate buffer	0.05
50 mM Potassium phosphate buffer	0.02
Buffer A **(Section 7.3)**	0.01
PDB	0.02

Table 7-2 Solubility of progesterone in various media.

7.5.4.1 Effect of progesterone concentration on induction of P450 11α

Induction at 16 hr into the incubation with 0.01 g/L progesterone resulted in undetectable activity of the conversion of progesterone to hydroxyprogesterone by HPLC (Perkin Elmer HPLC detector).

For detectable induction of P45011α the concentration of progesterone required was found to be at least 1 g/L, a concentration of 2 g/L resulted in excellent and reliable induction. An aqueous solution of 2 g/L Progesterone appears as a heterogeneous suspension.

7.5.4.2 Effect of induction time on biomass and P45011α activity

The production of P450 11α was induced by the addition of progesterone **(Osborne, 1990),** while *R. stolonifer* was in the growth phase, (16 hr after inoculation). Incubation of growing cells for 2 hr resulted in whole cells that had measurable P450 11α activity. Cells of *R. stolonifer* were harvested at 18 hr. The effect of induction time on the final biomass was not documented in Osborne's work, hence, the effect of induction time on the level of induction and the effect of biomass was determined in this work.

Progesterone was added to the cultures of *R. stolonifer* at different time intervals during the growth phase. After 18 hr the cells were harvested, washed, dried and weighed.

The addition of progesterone was found to reduce the final biomass of the culture **(Figure 7.4).** This reduction is typically 20 % (dwt) and the reduction is independent of

the time range tested. The activity of P450 11α was unaffected by the addition of progesterone early in the fermentation

7.5.5 determination of biomass

Wet weight of a 100 mL of culture of *R. stolonifer* was found to be 2.4 ±0.1 g (wwt). Dry weight analysis showed that ~6 % of the wet weight was biomass (dwt).

7.6 Conclusions and Discussion

From the results presented it is evident that the concentration of progesterone used to induce production of P450 11α and the concentration of progesterone in the bioconversion media has a strong influence on the hydroxylation of progesterone with 11α- hydroxylase in *R. stolonifer.*

The activity of P450 11α was found to be higher when higher amount of progesterone was used. The induction of progesterone 11α hydroxylase could be initiated in the growing phase and in the resting phase where no growth occurs. This indicates that the production of P450 11α hydroxylase activity is a highly evolved mechanism. In all cases a delay in activation was seen which could be a result of progesterone partitioning from the media in to the lipid membranes of *R. stolonifer.*

2 g/L Progesterone appeared as an un-dissolved suspension in buffer A. This solid suspension appeared to be essential in the induction of activity. Solid progesterone must partition into the bio-membranes of *R. stolonifer* to initiate activation. The ability to induce P450 11α activity at the start of the inoculation resulted in a higher experimental turnover.

This observation could be relevant in a production environment. Progesterone did exert some growth inhibition. The small reduction in final biomass also indicates that the progesterone in not highly toxic and adds to the evidence that the *R. stolonifer* has an efficient detoxification mechanism for the removal of this steroid.

7.6.1 Assay of progesterone 11α-hydroxylase activity

It was found that the concentration of progesterone could not be determined accurately using pipette sampling and this was attributed to the heterogeneous nature of the incubation media. It was found that the hydroxyprogesterone had a higher solubility in the aqueous phase and hence the rate of the hydroxylation reaction was determined by the production of hydroxyprogesterone **(Figure 7.5).** The concentration of progesterone used to induce P450 11α activity and the concentration of progesterone in the incubation media was found to have an effect on the initial rate of the reaction. A lag phase appeared when a higher concentration of progesterone was used in the incubation broth than used to induce P450 11α activity **(Figure 7.5).** *R. stolonifer* displayed P450 11α activity even when not induced within the fermentation. When the concentrations of progesterone in the induction period and during incubation were equal no lag phase was seen **(Figure 7.6).** It appears that the low level of progesterone (0.2 g/L) is insufficient to induce the hydroxylase activity to its maximum potential, and that further induction occurs during the assay.

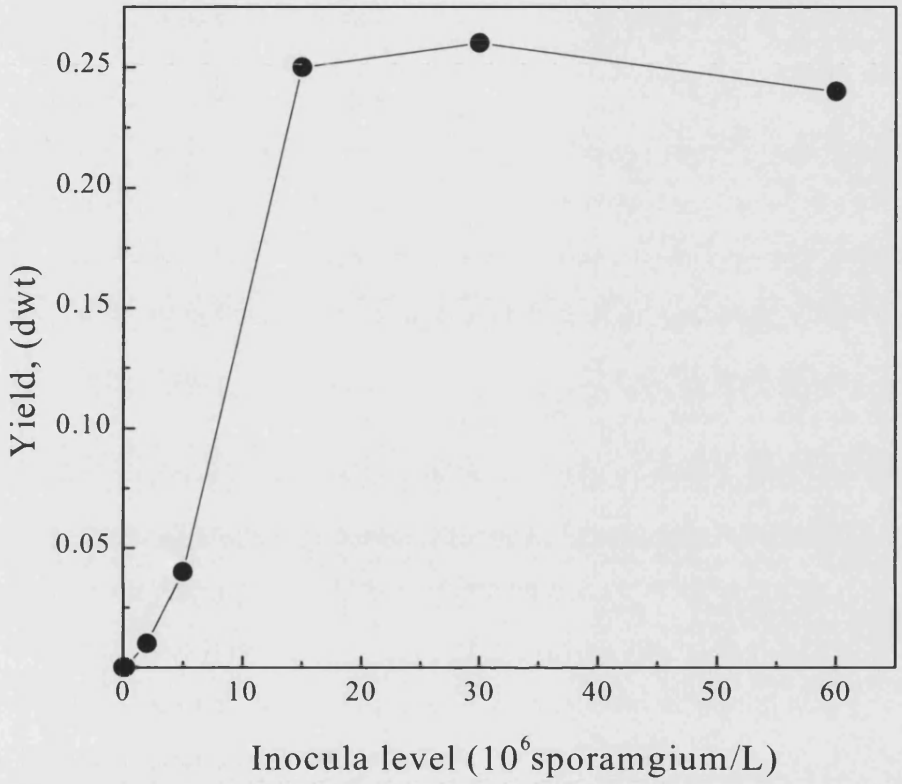


Figure 7-1 Comparison of final cell weight and spores inoculum levels. Results for *R. stolonifer* culture in of 24 g/L PDB (100m L), after 24 hr at 28 ° C and 200 RPM.

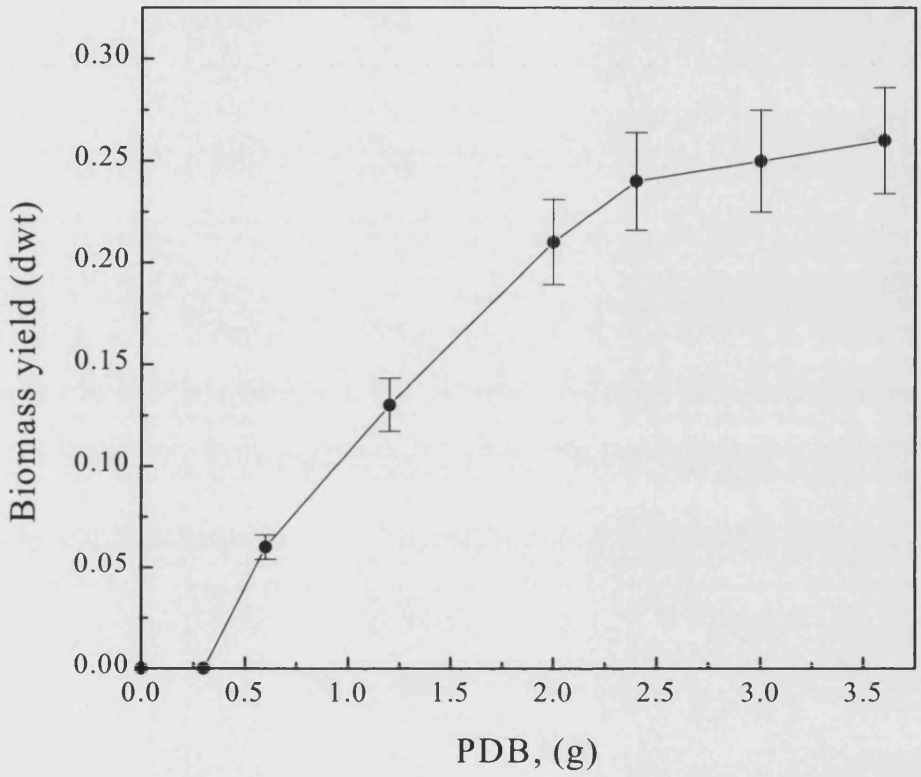


Figure 7-2 Effect of the concentration of PDB on the *R. stolonifer* yield. Yield is as expressed as the dwt compared with the weight of PDB. Data is from three 100 mL fermentations. Initial inocula concentration of $15x10^6$ sp./L was used.

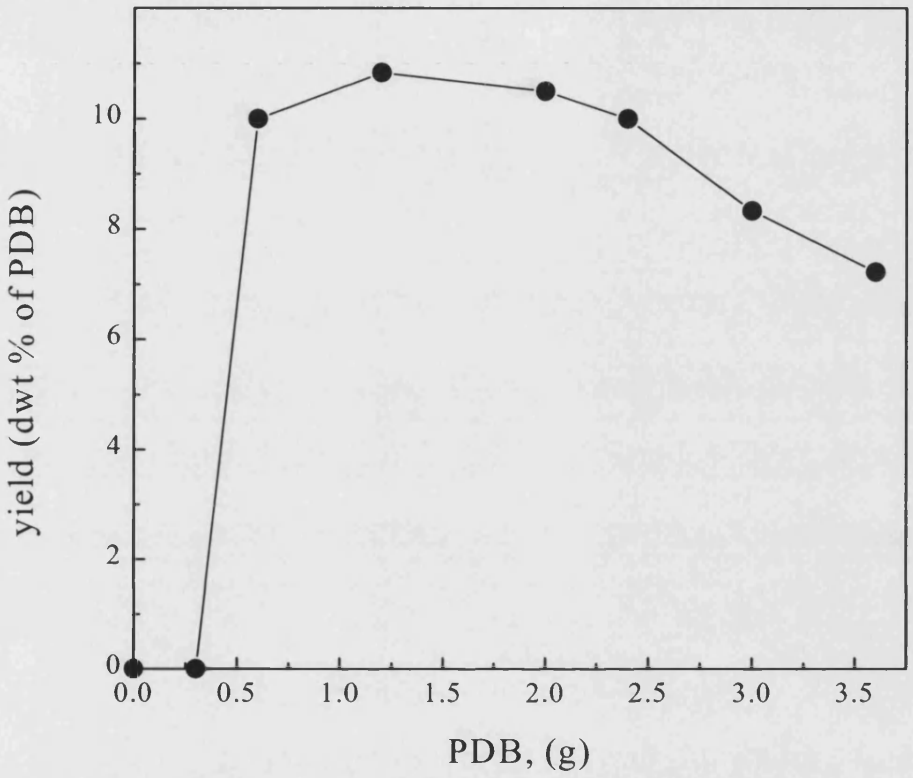


Figure 7-3 Effect of *R. stolonifer* yield, expressed as the dry cell weight as a percentage of PDB.

Initial inocula concentration of 15 x 10^6 sp./L.

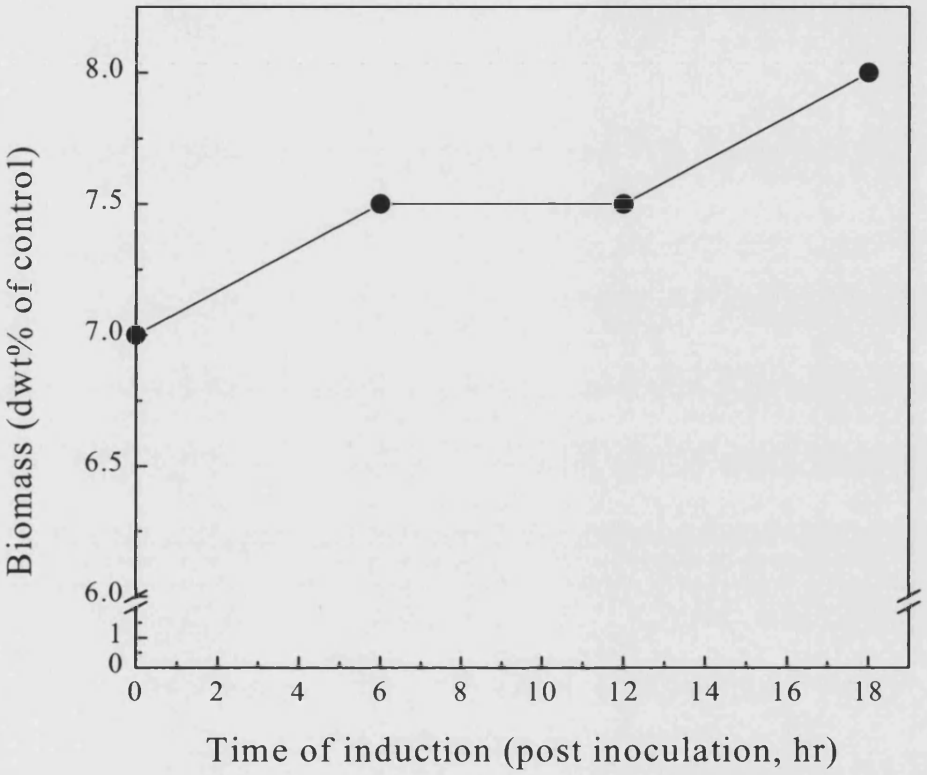


Figure 7-4 Effect of induction time with 2 g/L progesterone, on the biomass of *R. stolonifer* at 18 hr of incubation in PDB. Biomass is expressed, as dwt as a percentage of control were no progesterone was added.

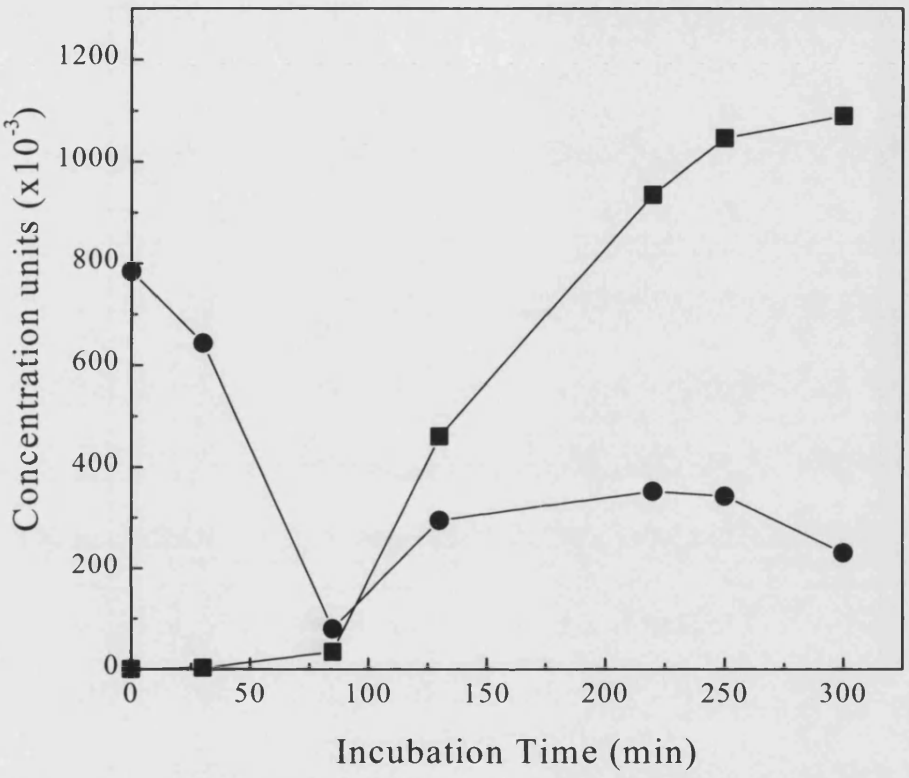


Figure 7-5 The Biotransformation of 2 g/L Progesterone to Hydroxy-progesterone.

Assay conditions: Incubation of *R. stolonifer* induced with 0.2 g/L progesterone. The biotransformation of progesterone (●), to hydroxyprogesterone (■) was followed by HPLC.

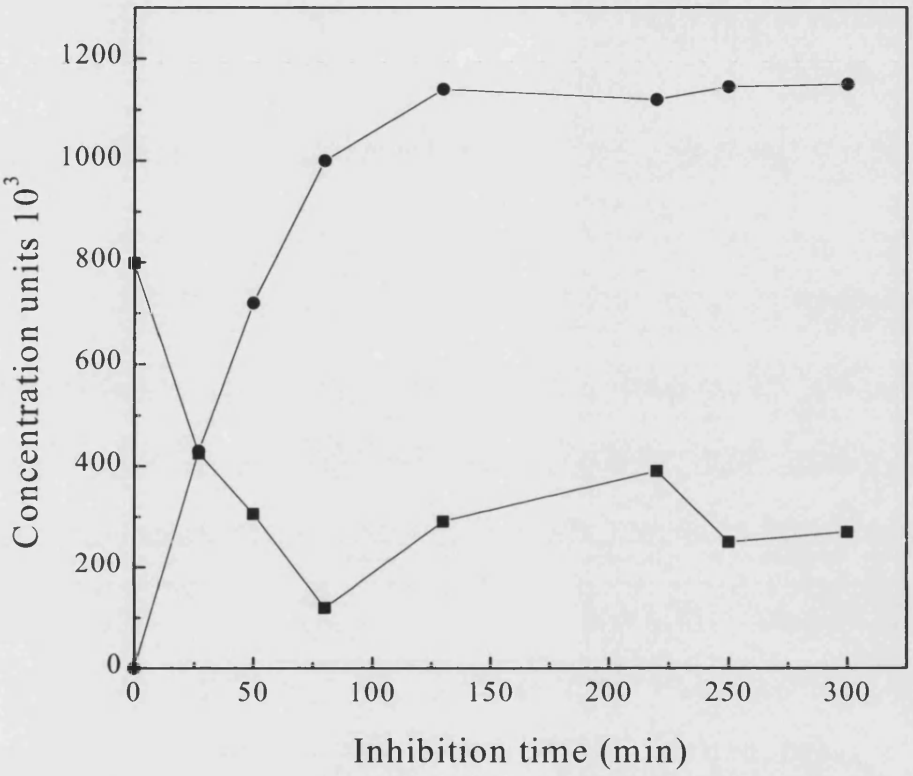


Figure 7-6 The Biotransformation of Progesterone to Hydroxy-progesterone. Assay conditions: *R. stolonifer* induced with 2.0 g/L of progesterone was incubated with 2.0 g/L of progesterone (■). Biotransformation to hydroxyprogesterone (●) was followed by HPLC

8. Inhibition of whole cells of *R. stolonifer* with *n*-alcohols

8.1 Abstract

(1) The specific hydroxylation of progesterone to 11α-hydroxyprogesterone was monitored in whole cells of *R. stolonifer*.

(2) The series of *n*-alcohols from methanol to octanol inhibits the initial rate of the reaction with increasing potency as the molecular weight increases.

(3) The ability to inhibit the reaction by 50% (IC_{50}) is limited to the first seven members of the homologous series.

8.2 Introduction

The specific hydroxylation of progesterone to 11α-hydroxyprogesterone can be catalysed by the monooxygenase system P450 11α in *R. stolonifer* **(Section 1)**, and is an example of a microbial biotransformation of a hydrophobic compound. The low solubility of substrate in the aqueous phase can cause mass transfer limitations and reduces the overall activity of the reaction. The use of methanol/aqueous co-solvent can be used to increase the catalytic activity, **(Osborne *et al.*, 1990).** Two-phase systems however are inhibitory and the *n*-alcohols from methanol to hexanol inhibits with increasing potency as the molecular weight increases **(Osborne *et al.*, 1992).** In that study the conversion of progesterone was determined after 24 hr. The effect of *n*-alcohols on the initial activity was not documented. The effect of solvent on K_m and V_{max} cannot be determined. In this investigation the initial rates of reaction will be documented and the effects of *n*-alcohols (nC1-nC6) on the rate of reaction will be determined.

8.3 Materials and Methods

Organism, growth conditions and induction were those documented earlier **(Section 7).**

8.3.1 Incubation of whole cells with alcohol

Harvested cells of *R. stolonifer* were re-suspended to a concentration of 10 g/L (wwt) in reaction mixture, containing 50 mM phosphate buffer pH 7.4, 2 g/L progesterone and various concentrations of *n*-alcohol to a final reaction volume of 20 mL. Incubations of whole cells and progesterone in alcohol solutions were performed in a shaker at 150 RPM, 20 °C. The incubation was followed by HPLC **(Section 7).**

8.4 Results

8.4.1 Incubation of whole cells with alcohol

8.4.1.1 Development

$MgCl_2$ and glucose were removed from the incubation buffer since the addition had no effect on activity over 24 hr in our preliminary experiments. The initial rate of reaction was measured over a period of 4 hr. When the concentration of progesterone used in incubation experiments was higher than the concentration used in induction experiments there was an extended period of low activity in the reaction vessel. When the incubation and induction concentrations were identical there was an immediate production of hydroxyprogesterone.

8.4.2 Inhibition with methanol

Each alcohol was added to the incubation medium in turn and the hydroxylation reaction was followed. The effect of alcohols on the rate of reaction was repeated for methanol to *n*-hexanol **(Figure 8.1).** The IC_{50} values were estimated when the percentage enzyme activity remaining is plotted against the Log_{10}.

8.5 Discussion

From the results presented in **Figure 8-2** it is evident that the chain length of the alcohol has a strong influence on the rate of hydroxylation of progesterone with 11α- hydroxylase of *R. stolonifer*. Values for IC_{50} and the Log P of the *n*-alcohol are highly correlated **(Figure 8-3).** This confirms the result given earlier. The historical data shows a tendency to underestimate the IC_{50} concentrations for propanol to octanol. Indeed *n*-octanol was unable to inhibit the reaction to 50 % but a value of 5.4 mM is indicated. This is higher than the solubility limit in pure water, 3.2 mM. Methanol and ethanol however, were underestimated.

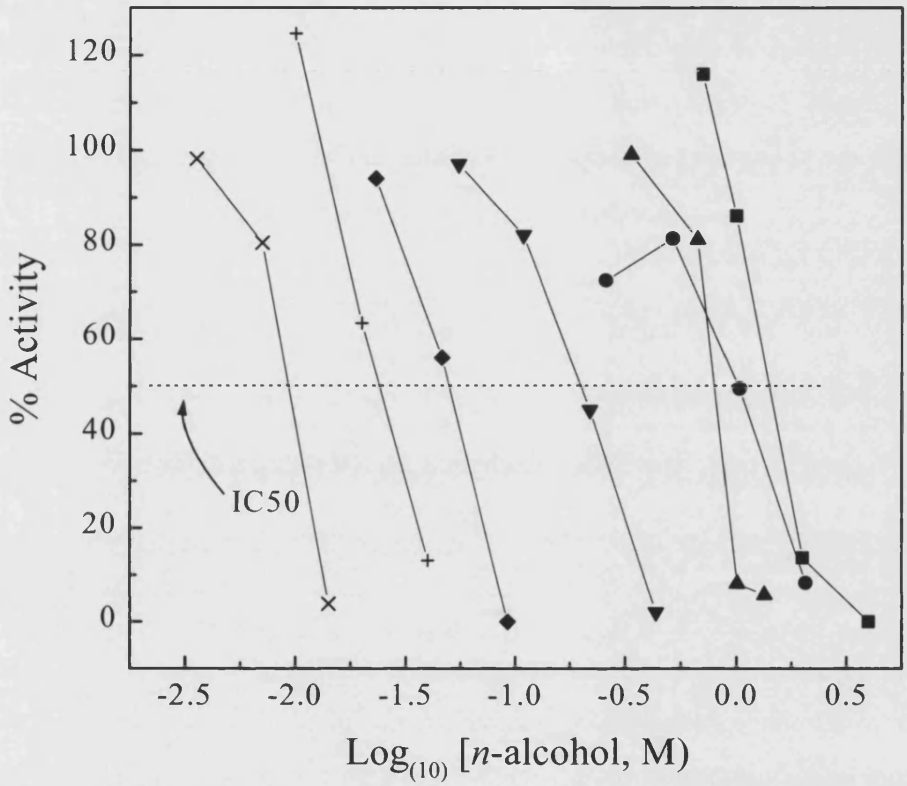


Figure 8-1 Graph to show the effect of the total concentration of *n*-alcohols in the system on the activity of P450 11α.

Activity was measured as the rate of progesterone conversion to hydroxyprogesterone and was recorded as a percentage of that in the absence of alcohol. (■) methanol, (●) ethanol, (▲) propanol, (▼) butanol, (♦) pentanol, (+) hexanol, (X) heptanol.

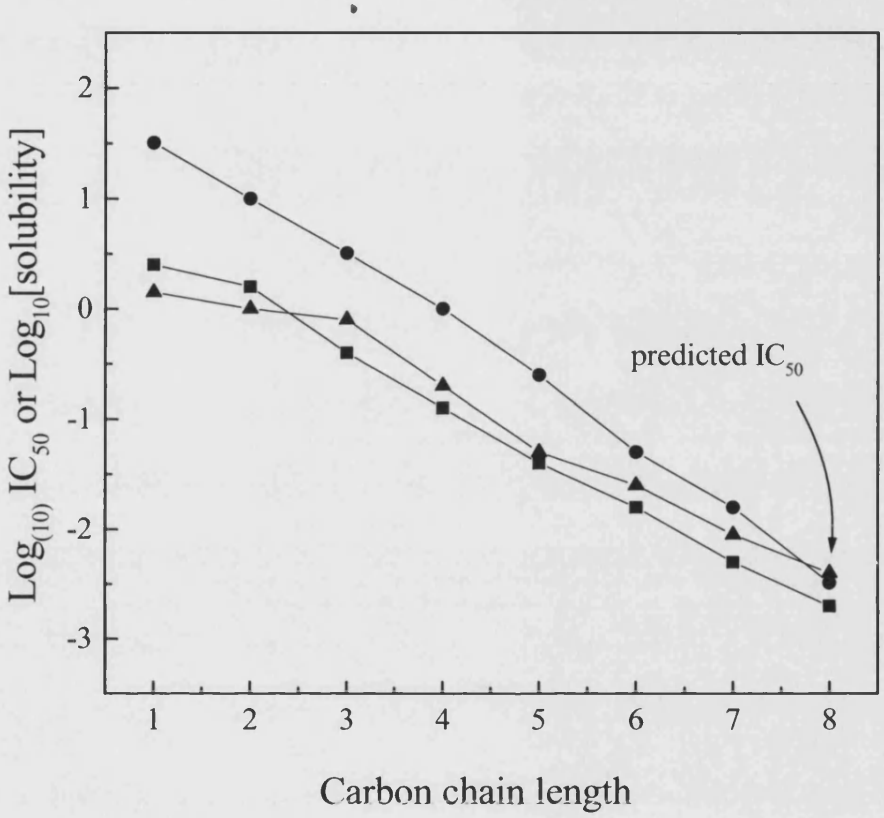


Figure 8-2 Concentration of alcohol added to the system to achieve 50% inhibition of the *R. stolonifer* hydroxylase.

(■) historic IC_{50} (Osborne *et al.*, 1990). (▲) experimental IC_{50} (●) solubility in moles/L Where the alcohol is miscible with water the data shows the molar concentration of the neat alcohol.

9. Partitioning of solvents into whole cells of *R. stolonifer*

9.1 Abstract

(1) The partitioning of *n*-alcohols in whole cells of *R. stolonifer* was monitored using the methods developed previously **(Section 3).**

(2) Alcohols from methanol to octanol were found to associate with cellular membranes of *R. stolonifer* to a fixed ratio.

(3) This amount of solvent was found to be similar for all the alcohols tested.

9.2 Introduction

The initial rate of the hydroxylation of progesterone to 11α hydroxy-progesterone by whole cells of *R. stolonifer* has been inhibited with *n*-alcohols. The ability to inhibit the reaction increases with the chain length of the alcohol **(Section 8).** The aqueous concentration of *n*-alcohol required to inhibit the reaction by 50 % (IC_{50}) correlates well with the *n*-octanol water partition coefficient (Log P) of each of the *n*-alcohols in the homologous series from methanol to heptanol **(Section 8).** The Log P and the membrane partition coefficient (P_{mem}) can also be related **(Collander, 1951).** Exact determinations of inhibiting solvents that are associated with the biological membrane phase however, to this date have not been obtained experimentally. They have been inferred from Log P and Log P_{mem} data and assume that they are related by a linear function **(Seeman, 1972)** or some power function **(Collander, 1951).** In this section exact concentrations of *n*-alcohols will be determined for whole cells of *R. stolonifer* using the solvent assay developed **(Section 3).** They will be correlated with the aqueous concentration data (IC_{50}) obtained earlier **(Section 8).**

9.3 Materials

Buffer A and liquid media PDB was used to cultivate cells of *R. stolonifer* as described earlier **(Section 7.3)**.

9.3.1 Partitioning Media

1.25 M methanol, 1 M ethanol, 0.79 M propanol, 0.20 M butanol, 0.05 M pentanol, 0.04 M hexanol and 0.0079 M heptanol were used to partition into whole cells of *R. stolonifer*. These were the apparent IC_{50} concentrations of the alcohols examined and are the total concentrations of the alcohols added to the incubation mixtures.

9.4 Methods

9.4.1 Organism, Growth and harvest conditions

Growth procedures were used as reported earlier **(Section 7)**. A final fermentation step in 2 L baffled shake flasks was used to produce the required cell biomass. Harvested cells were obtained using nylon mesh **(Section 7).**

9.4.2 Incubation of whole cells with *n*-alcohols

0.4 g (wwt) of fresh *R. stolonifer* was added to partitioning media (40 mL). This is the same cell concentration (10g/L) as was used in the measurement of progesterone hydroxylation. Samples (1 mL) were taken at time intervals and spun in a mini centrifuge (113 lab centrifuge Sigma, Germany) for 2 min. at 13,000 RPM.

9.4.3 Solvent assay

Samples of the supernatant were added to the solvent test mixture **(Section 3.2)**, and the reaction recorded by luminescence **(Section 3.1)**.

9.5 Results

9.5.1 Effect of time on the partitioning of *n*-alcohols tested.

9.5.1.1 Concentration of alcohol in the aqueous phase.

The concentration of alcohol in the aqueous phase was determined by using standard curves obtained earlier for the inhibition of luciferase by *n*-alcohols **(Section 3.3).**
The results can be seen below **(Table 9-1).**

Carbon chain Length	**[$ROH_{(total)}$] mM**	**[$ROH_{(aq)}$] mM**	**Amount per g of wet cells (10^{-3} Moles)**
1	1250	1200	---
2	1000	985	3.1
3	790	780	6.1
4	200	195	3.1
5	50	46	2.5
6	40	37	1.5
7	7.9	6	1.2

Table 9-1 The partitioning of *n*-alcohols into cellular membranes *of R. stolonifer.*

From this data it is possible to calculate the concentration of solvent taken up by each gram of wet cells. This appears to be about 3 mmoles, and to be remarkably consistent over the range of alcohols from ethanol to heptanol.

10. Statistical Analysis

A comparison of the inhibition of progesterone hydroxylation by Cytochrome P450 11α in whole cells of *R. stolonifer* **(Figure 8-1)** and the inhibition of sodium laurate hydroxylation by Cytochrome P450 BM3 in *B. megaterium* **(Figure 6-4)** was made in this section. Regression analysis was used to determine the statistical significance of the effect of alcohols on both hydroxylation reactions, and to determine whether there was any difference between the response of the two systems to the alcohol concentrations. This section explored the statistical methods used to obtain confidence in the final data and the conclusions obtained from the data.

10.1 Statistical analysis of errors

A normally distributed population is characterised by a mean and a standard deviation. The first, which is the population mean, centres the population, and the second, which is the standard deviation of the population, measures the spread around the centre.

In an experimental system an attempt is made to estimate both the mean and the standard deviation. Accurate values of either cannot be obtained even if the measurements are extremely accurate and precise because they are only a sample of the population. The sampling itself introduces errors, which cause the estimated values, the sample mean and standard deviation, to differ from the actual values, the population mean, and standard deviation.

A sufficiently large number of samples needed to be taken to ensure that the sampling errors were reduced to an acceptable level. For example if the sample were to contain just two measurements both might be to one side of the population mean, either high or low. The values could also be widely separated or perhaps close together. Wherever they lie there is clearly a high probability that they will provide a skewed estimate of the population mean and the standard deviation.

10.1.1 Standard error of the mean, (SEM)

The standard error of the mean (SEM), that is the error associated with the estimate of the mean, can be calculated from the standard deviation (SD) of the measurements in the sample, which is taken. It equals $SD/\sqrt{n}$ where n is the number of measurements, which make up the sample. The SEM is the figure, which should follow the +/- sign (ie Mean +/- SEM), and should be used for the error bars. It is also used to compare means in Student's t-test to check for the significance of a difference between the calculated means of two different populations.

10.1.2 Analysis of trends

Due to the instability of cytochrome P450 from *B. megaterium* in solution it was essential to prepare fresh samples of the enzyme from fermentation flasks and only one assay reading was recorded. Each of the *R. stolonifer* inhibition experiments could be carried out over many hours, but due to the time constraint it was decided to obtain only one estimate of the IC_{50} for each alcohol. The data for as many alcohols as possible was obtained, so as to fully understand the relationship between solvent inhibition and alcohol chain length.

Although each sample point represents a single measurement the data as a whole shows consistent trends **(Sections 10.2.1 and 10.2.2).** Where trends are recorded the data from neighbouring estimates ought to reinforce, because the sampled populations should be related. It is then possible to estimate the errors for the overall experiment even if only one measurement is made at each point. However, to estimate the errors it is necessary to make some assumptions about how the variables are related.

10.1.3 Linear Regression analysis (Straight line relationship)

All of the data in these experiments was assumed to be related through linear trends. Where these are not immediately apparent some consistent transformation of the data was

applied. The assumptions made are described later in the sections that describe the inhibition data itself.

Microcal Origin 4.1 was used to perform the linear regression analysis. The data was set up in an Origin data sheet, with the independent variable, for example the alcohol concentration, on the x-axis. The dependent variable, the one that was measured, for example the degree of inhibition, is plotted on the y-axis. Origin was used to fit a straight line to this data. The calculation sheet provided values for the correlation coefficient (r), and square root of the residual variance (SD).

The correlation coefficient (r), was used to check that the regression coefficient (the slope of the straight line) is significantly different from 0. A table of values for r, with n-2 degrees of freedom, where n is the number of data points was used for this purpose.

The 95% confidence limits for the individual data points were calculated from the value of SD according to **Equation 10-1**. Microsoft Excel was used for this purpose.

$$y_p \pm t \cdot SD \cdot \left(1 + \frac{1}{n} + \frac{(x_p - \bar{x})^2}{\sum x^2 - \frac{(\sum x)^2}{n}} \right)^{0.5}$$

Equation 10-1 Equation used to determine the 95% confidence limits

where x_p is the value of x for which the predicted value of y is required, and Student's t has a value appropriate to the 95% confidence limit (ie $p<0.05$) with n-2 degrees of freedom. Note that the variance of y about the regression line becomes larger the further you move away from $\bar{x}$, the mean value of the independent variable.

The correlation coefficient and the 95% confidence limits contain the accumulated errors from all of the data used in their calculation.

10.2 Results

10.2.1 B. megaterium

In each inhibition experiment with the separate alcohols we have used a range of concentrations and measured the respective hydroxylation activity **(Figures 5-6 & 7-1).** Appropriate alcohol concentrations were chosen to ensure that the levels of inhibition of the hydroxylation varied between 10 and 90%.

10.2.1.1 Effect of alcohols on the activity of progesterone hydroxylase activity in *B. megaterium* - Calculation from complete data set

Depending on the alcohol we have up to 8 data points from which to obtain a linear trend with the aim of measuring the IC_{50} for each alcohol. The mathematical function that describes that line is $Y = BX + A$ where Y is the % enzyme activity, X is the $Log_{(10)}$ of the alcohol concentration, B is the slope of the line and A is the intercept on the Y axis. The concentration that produces 50% inhibition can then be obtained by calculating (50-A)/B.

Initially all of the experimental data obtained for was tabulated in Origin and the linear function $Y = A + BX$, the correlation coefficient, the standard deviation and the probability were calculated for each alcohol **(Figure 10-1, Table 10-1).**

The slopes of the lines **(Figure 10-1)** are all negative. But they are different across the range of alcohols tested. The spacing across the x-axis was also irregular, but as the chain length of the alcohol increases the intercept on the IC_{50} value increases.

The hexanol plot gives us a very low slope. This is because we are working at the limit of solubility and prevents us from obtaining experimental values of inhibition below about 75%

In general this variation in the slopes is not what we would have expected from the original Osborne data **(Osborne *et al.*, 1990)** where the slopes are much more regular **(Figure 10-2).**

However a plot of the remaining hydroxylation activity against the alcohol concentrations actually produces a set of sigmoidal curves **(Osborne *et al* 1990)** and data taken from the extremes of high and low inhibition is not really not appropriate in fixing the mid point inhibition based on linear trends.

Alcohol	N	A	B	Corr Coef	St. dev	P	$Log_{(10)}$ [Alc]
Methanol	8	41	-54	-0.989	4	<0.0001	-0.164
Ethanol	9	18	-75	-0.978	7	<0.0001	-0.422
Propanol	7	-1	-57	-0.976	7	<0.0001	-0.880
Butanol	6	-33	-74	-0.936	12	0.006	-1.126
Pentanol	6	-21	-59	-0.911	11	0.011	-1.212
Hexanol	3	27	-25	-0.985	2	0.110	-0.927

Table 10-1 Results of the regression analysis on the complete data set for the samples of *B. megaterium*. The data records a linear fit (Y = A + BX) to the relationship between the percentage activity and $Log_{(10)}$ alcohol for each alcohol.

1.2.1.2 Effect of alcohols on the activity of progesterone hydroxylase activity in *B. megaterium* - Calculation from reduced data set

When only the data covering inhibition from 20% to 80% is plotted the spacing and slope of the lines are much more regular, however the hexanol cannot be plotted. We would expect the spread of the data about the fitted line to reduce when using the restricted data set because of the sigmoidal nature of the inhibition data. Comparing the values of the

standard deviations in the two plots **(Figure 10-1 and Figure 10-3)** can see this. There is also an increase in the correlation coefficients.

When comparing the spread of the data around the centre, the average standard deviation for the complete sample set is 7.2 whereas the average standard deviation for the reduced set is 4.2. This would suggest that the reduced set is more representative of a straight line from which the IC_{50} values can be calculated.

Alcohol	N	A	B	Corr Coef	St. dev	P	Log(10)[Alc]
Methanol	6	41	-61	-0.955	4	0.003	-0.147
Ethanol	7	18	-84	-0.992	3	0.000	-0.376
Propanol	4	-13	-77	-0.985	5	0.015	-0.814
Butanol	3	-183	-219	-0.995	3	0.060	-1.063
Pentanol	5	-66	-95	-0.936	8	0.019	-1.211

Table 10-2 Results of the regression analysis on the data set within the limits 20% to 80% inhibition for the samples of *B. megaterium*. The data records a linear fit (Y = A + BX) to the relationship between the percentage activity and $Log_{(10)}$ alcohol for each alcohol.

10.2.1.2 Trend of inhibition of *B. megaterium* hydroxylase.

The derived values **(Table 10-3)** of the $Log_{(10)}$ Alcohol at 50% inhibition can then be plotted against the carbon chain length and a new linear trend fitted. The dotted lines show the 95% confidence limits calculated according to the equation shown **(Section 1.1.3).**

All of the errors in the experiment are accumulated in this line **(Figure 10-4).** The advantage of setting up the data in this way is that it allows data from all of the alcohols to

be used to measure the errors, even though each assay shown **(Figure 10-1)** was only performed once.

The dotted lines show the 95% confidence limits for the correlation. If you know the number of carbon atoms in the alcohol then a new estimate of the concentration of alcohol giving 50% inhibition should lie within the dotted envelope with a probability ≥ 95%. Another way of looking at this is to say if we were to make another estimate of the alcohol concentrations at 50% inhibition and the value were to fall outside these dotted lines there is only a 5% chance that the difference would be due to random variation. In fact the new estimate which fell outside of these confidence limits would be significantly different from the value which the straight line predicts.

x^2	Alcohol (x)	Log_{10}[alc] (y)	Mean	Error	5% upper	5% lower
1	1	-0.147	-0.1592	0.434	0.275	-0.593
4	2	-0.376	-0.4407	0.352	-0.088	-0.793
9	3	-0.814	-0.7222	0.325	-0.397	-1.048
16	4	-1.063	-1.0037	0.352	-0.651	-1.356
25	5	-1.211	-1.2852	0.434	-0.851	-1.719

Table 10-3 Tabulated values for the regression line for the relationship between $Log_{(10)}$ IC_{50} and carbon chain length for *B. megaterium*

10.2.2 R. stolonifer

10.2.2.1 Effect of alcohols on the activity of progesteone hydroxylase activity in ***R. stolonifer***

The analysis of *B. megaterium* was replicated for *R. stolonifer* using all data points **(Section 8.5.2).** The results are tabulated in **(Table 10-4 and 10-5).**

Alcohol	N	A	B	Corr Coef	St. dev	P	Log(10)[Alc]
Methanol	4	84	-162	-0.961	19	0.039	0.2117
Ethanol	3	48	-121	-0.997	4	0.048	-0.0158
Propanol	4	26	-172	-0.926	22	0.074	-0.1381
Butanol	4	-31	-107	-0.981	10	0.019	-0.7504
Pentanol	3	-158	-156	-0.994	7	0.070	-1.3354
Hexanol	3	-249	-186	-0.998	4	0.036	-1.6086
Heptanol	3	-278	-157	-0.941	24	0.220	-2.0814

Table 10-4 Results of the regression analysis on the complete data set for the samples of *R. stolonifer*. The data records a linear fit (Y = A + BX) to the relationship between the percentage activity and $Log_{(10)}$ alcohol for each alcohol.

The actual values for the total alcohol concentrations which must be added to the system to cause 50% inhibition are for methanol 1.63 M, ethanol 0.96 M, propanol 0.73 M, butanol 0.17 M, pentanol 46 mM, hexanol 21 mM, and heptanol 8 mM. These values are not very different from those used to estimate the concentration of alcohols that remain in the aqueous phase after partitioning into the cells of *R. stolonifer* **(Section 9).**

This is the final result of the effect of alcohols on the hydroxylase in *R. stolonifer*. The dotted lines show the 95% confidence limits for the correlation. If the number of carbon atoms in the alcohol is known, then the concentration of alcohol giving 50% inhibition should be found within the dotted envelope with a probability >95%.

x^2	Alcohol (x)	Log_{10}[alc] (y)	Mean	Error	5% upper	5% lower
1	1	0.212	0.390	0.606	0.996	-0.216
4	2	-0.016	-0.012	0.532	0.520	-0.545
9	3	-0.138	-0.415	0.488	0.073	-0.903
16	4	-0.750	-0.817	0.473	-0.344	-1.290
25	5	-1.335	-1.219	0.488	-0.731	-1.707
36	6	-1.609	-1.621	0.532	-1.089	-2.154
49	7	-2.081	-2.024	0.606	-1.417	-2.630

Table 10-5 Tabulated values for the regression line for the relationship between $Log_{(10)}$ IC_{50} and carbon chain length for *R. stolonifer*

10.3 Comparison of data from *B. megaterium* & *R. stolonifer*

The complexity of setting up the experimental conditions limited the number of trials that could be run. The two linear trends need to be compared to discover if there is a difference between the inhibition of the progesterone and sodium laurate hydroxylation reactions. Because of the limited data set, an analysis of the errors is required. We need to determine if the two lines are statistically different or similar.

10.4 Statistical analysis of the difference in slopes.

The analysis that Origin 4.1 gives us, does not directly compare the slopes of the two lines. The real question is whether the slopes (trends) of the two lines are different.

Simple linear regression analysis (above) was used to characterise the inhibition data for both *B. megaterium* and *R. stolonifer* separately. However multiple regression analysis can be used to compare the two sets of data and we have performed this analysis according to the techniques set out in section 11.10 of **(Wackerly *et al.*, 1995)** and using the worked example 11.79 whose solution is set out in **(Kincaid, 1996).**

The primary X and Y matrices were set up as below and the β matrix was derived directly from the simple regression analysis provided by Origin 4.1.

$$\mathbf{Y}=\begin{vmatrix} -0.147 \\ -0.376 \\ -0.814 \\ -1.063 \\ -1.211 \\ 0.212 \\ -0.016 \\ -0.138 \\ -0.750 \\ -1.335 \end{vmatrix}$$

$$\mathbf{X}=\begin{vmatrix} 1 & 0 & 1 & 0 \\ 1 & 0 & 2 & 0 \\ 1 & 0 & 3 & 0 \\ 1 & 0 & 4 & 0 \\ 1 & 0 & 5 & 0 \\ 1 & 1 & 1 & 1 \\ 1 & 1 & 2 & 2 \\ 1 & 1 & 3 & 3 \\ 1 & 1 & 4 & 4 \\ 1 & 1 & 5 & 5 \end{vmatrix}$$

$$\beta = \begin{vmatrix} 0.1223 & \beta_0 \\ 0.6697 & \beta_1 \\ -0.2815 & \beta_2 \\ -0.1207 & \beta_3 \end{vmatrix}$$

Where;

β_0 = Slope for *B. megaterium*

$\beta_0 + \beta_1$ = Slope for *R. stolonifer*

β_2 = Intercept for *B. megaterium*

$\beta_2 + \beta_3$ = Intercept for *R. stolonifer*

SSE=	0.0519
s=	0.0930
n-(k+1)=	6
t=	2.9005

0.025>p>0.005

The analysis yields (above) a sum of the squares of the errors (SSE), and a standard deviation (S), shown. From this data a Student's t value for the difference in the slopes at 6 degrees of freedom (n-(k+1)) is calculated as 2.9, which is significant at a 5% probability.

10.5 Discussion.

It has been shown that the 2 slopes **(Figure 10-3)** are statistically different **(Section 10.3)**. Therefore the partitioning effect alcohols in the two measured systems are significantly different.

Because of experimental limitations we are comparing a system with soluble enzyme and a

system with membrane bound enzyme within a whole cell extract so we are measuring partitioning into the bulk membrane and not just the membrane associated with the enzyme. The partitioning of alcohol into the bulk membrane and the membrane associated with the enzyme may be different. We cannot then reach any conclusion about the comparison of partitioning of alcohols into membrane bound enzyme using a soluble enzyme as a control.

We can however use this simple test as a reference point for the analysis of membrane bound enzyme against a soluble control. If in future the experimental limitations found in this system can be overcome or a different system tested then the original hypothesis can be more fully tested.

At this stage we have developed an analytical tool for quickly and reliably measuring the partition coefficients and gone some way to test the original hypothesis.

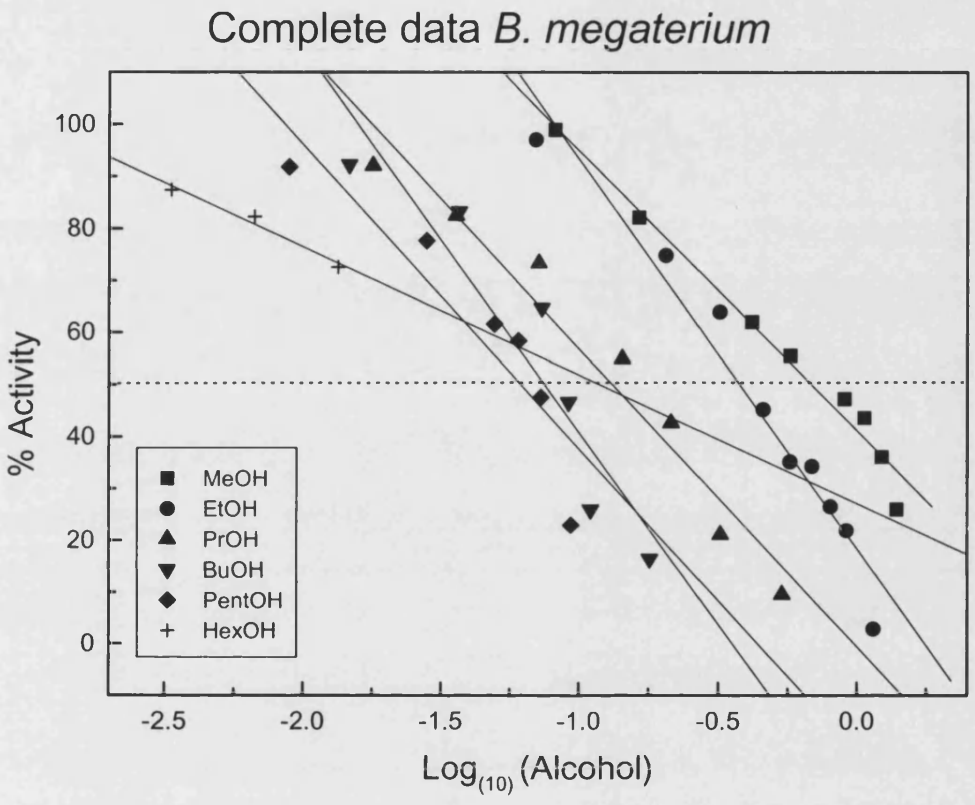


Figure 10-1 Inhibition of P450 BM3 by various alcohols from methanol to hexanol. The percentage activity remaining is plotted against $Log_{(10)}$ alcohol concentration. All data points are included in the plot

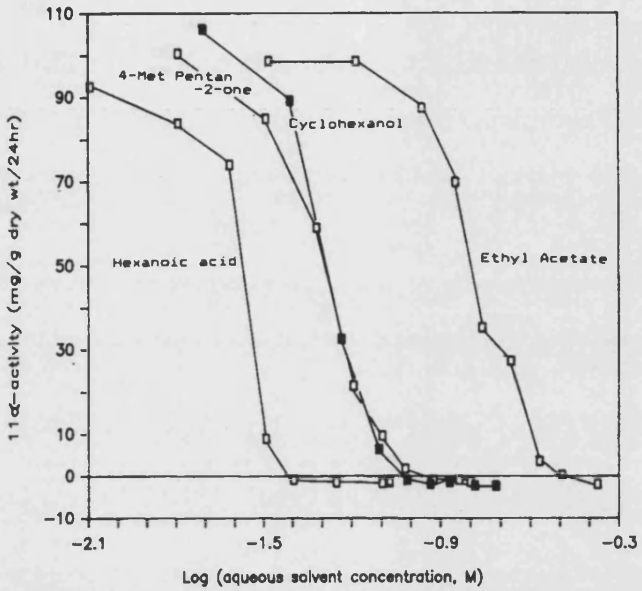


Figure 1 (a) 11α-Hydroxylase activity of *R. nigricans* in eight primary alcohol–aqueous cosolvent and two-liquid phase systems as a function of the logarithm of the solvent concentration in the aqueous phase. C1, C2, etc. refer to the number of carbon atoms of the alcohol. (b) 11α-Hydroxylase activity of *R. nigricans* in four organic–aqueous two-liquid phase systems as a function of the logarithm of the solvent concentration in the aqueous phase

Figure 10-2 Original Osborne data for the 11α-Hydroxylase activity of *R. nigricans* in eight primary alcohol-aqueous cosolvent and two-liquid phase systems as a function of the logarithm of the solvent concentration in the aqueous phase.

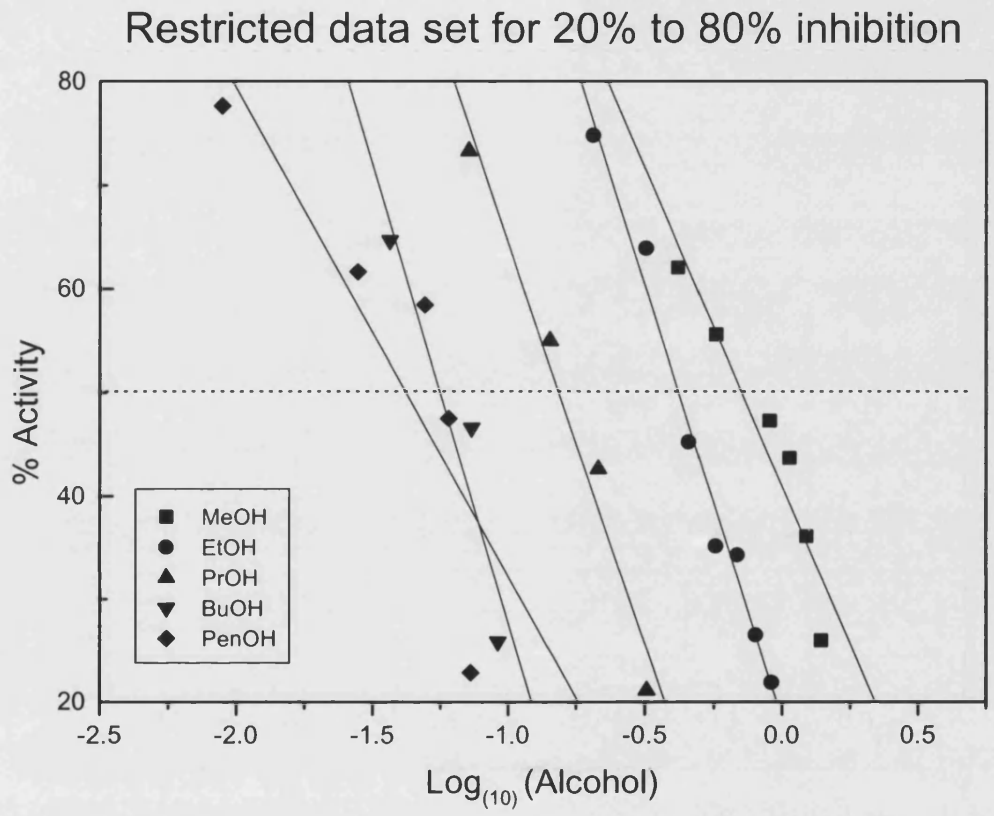


Figure 10-3 Inhibition of P450 BM3 by various alcohols from methanol to hexanol. The percentage activity remaining is plotted against $Log_{(10)}$ alcohol concentration. Only data between 20% and 80% inhibition is included.

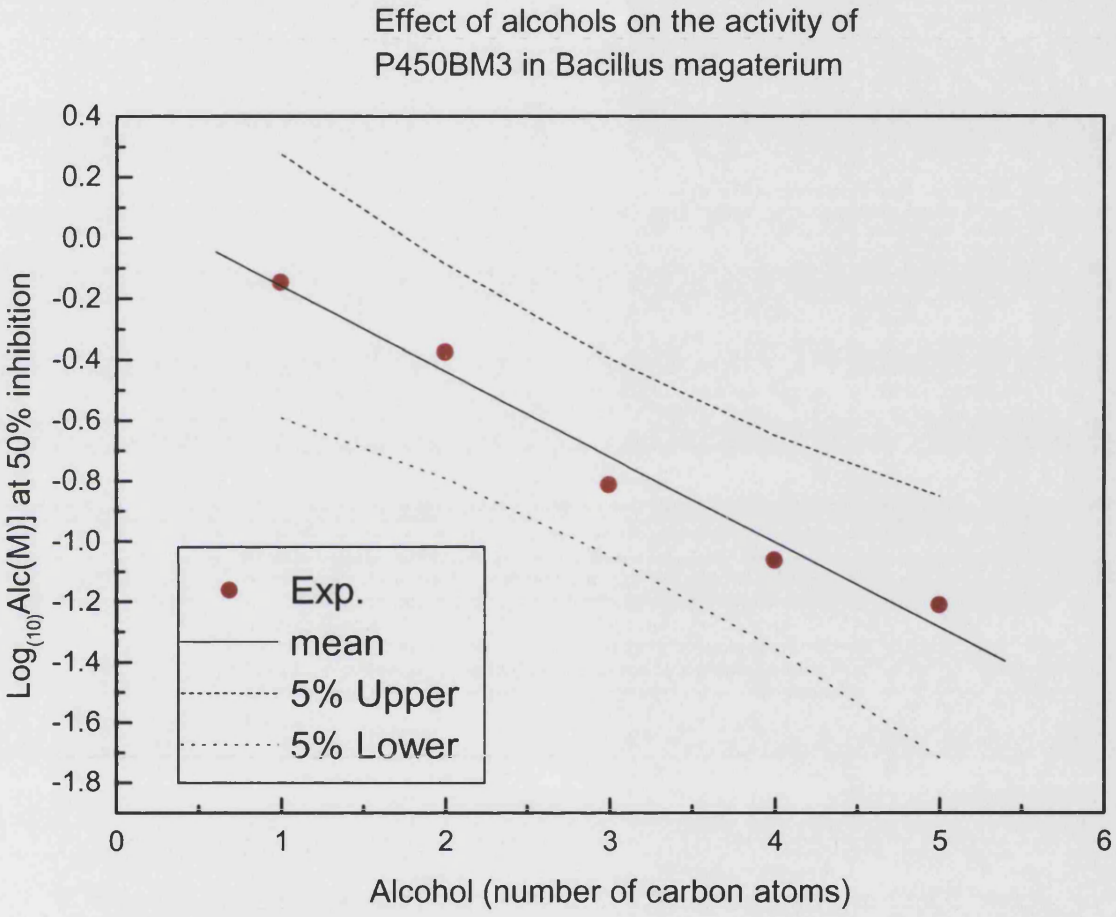


Figure 10-4 Regression line for the relationship between $Log_{(10)}$ IC_{50} and carbon chain length for *B. megaterium*.

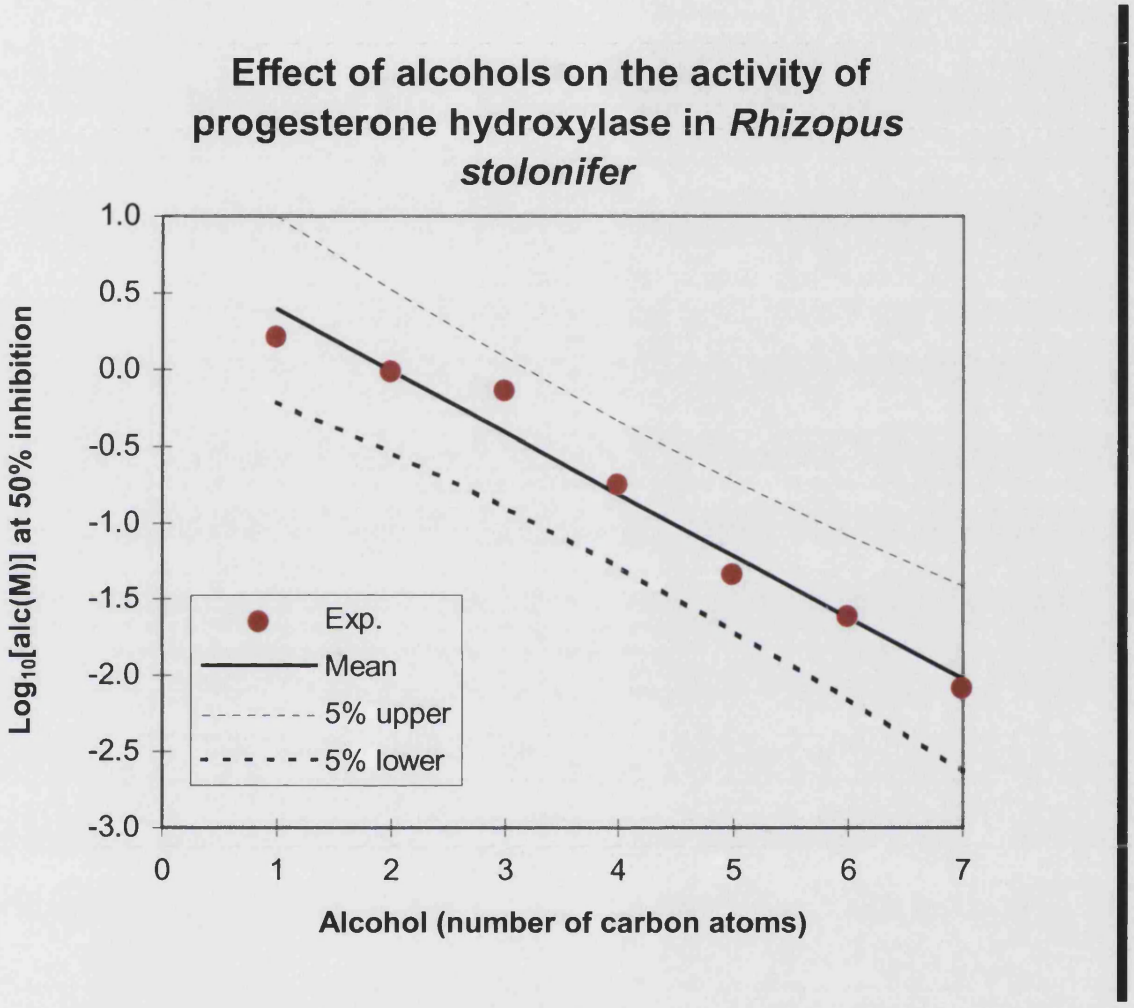


Figure 10-5 Effect of alcohols on the activity of progesterone hydroxylase in *R. stolonifer*)

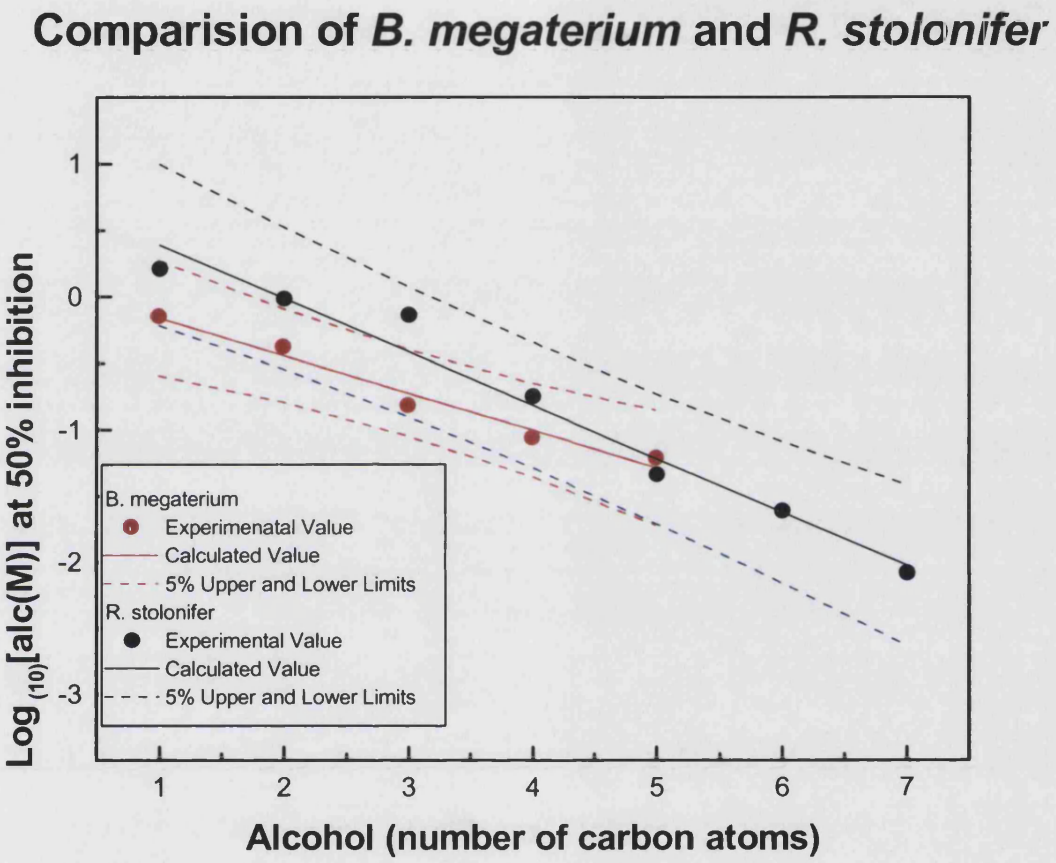


Figure 10-6 Regression analysis for the inhibition of P450 BM3 and P450 11α from Figures 10-4 and 10-5.

11. Discussion

11.1 Developing the solvent partitioning assay

The first of the experimental aims was to develop an assay technique to measure the partitioning of an organic solvent into the biological membranes of microorganisms. Ideally the measurement should be made without separating the two phases, because at high partition coefficients the contamination caused by a small amount of membrane in the aqueous phase would lead to large errors in the analysis. The method should report only on the aqueous phase of the system. This should be possible with the luciferase assay, as demonstrated **(Franks & Lieb, 1986).** Other assays are also available but they report on both the solvent associated with the membrane and solvent in the aqueous phase.

Once the luciferase assay is set up, it does provide, as demonstrated by **(Franks & Lieb, 1986)**, a direct report on the aqueous concentration of the solvents. It's applicable to a wide range of solvents, requiring only that the necessary standard curves be established for each of the solvents used. Other enzymes might be used in a similar fashion, but the light output form the luciferase assay is easy to measure directly.

There were some experimental problems with the setting the assay that were not initially envisaged. More time was spent on this part of the project but it was worth the effort because of the stable assay system that was developed, which was able to measure the aqueous concentrations of primary alcohols from methanol to pentadecanol, and alkanes from hexane to pentadecane.

11.2 Obtaining and comparing similar enzyme types

The second aim was to compare the effect of organic solvents on a soluble and a membrane bound enzyme. Cytochrome P450 was chosen because it is convenient and is available in both forms. We compared membrane bound Cytochrome P450 11α

hydroxylase from whole cells of *R. stolonifer* and soluble Cytochrome P450 BM3 from *B. megaterium*. The ideal enzyme for comparison would have been one that was available both in a soluble and in a membrane bound form. Were not able to compare a series of membrane bound enzymes as we initially forecast for the work.

11.3 Measuring Partition Coefficients

The ability of a solvent to inhibit the activity of a membrane-bound enzyme is related to two attributes. These are its solubility in water and its distribution ratio or partition coefficient between the membrane and the aqueous phases. The partition coefficient is defined as the ratio of the concentration of solvent in the aqueous phase and the concentration of solvent in the second phase. The second phase in this case being the biological membrane. Both the partition coefficient and solubility must be sufficiently high so that the solvent can deliver enough molecules from the aqueous phase into the biological membrane to reach the concentration required for inhibition.
For an accurate determination of solvent concentration in the membrane, the volume of the membrane phase and the partition coefficient must be known. Partition coefficients cannot easily be measured because we don't know the volume of the membrane phase. Since biological membranes are a complex mixture of lipid and water, the measured volume of hydrated membrane would be an overestimate of the true volume of the unhydrated lipid.

Moreover biological membranes that have specific functions may have different structures that support for that function. Specific membranes may have a specific affinity for a particular organic solvent. Different membranes are likely to have different partition coefficients, and the concentration of solvents in each of them will be different, but the aqueous concentration will reflect this mixture, because all of the phases are in equilibrium.

For this reason the dissolution of alcohols and other solvents in cellular membranes is usually predicted by the use of octanol/water partition data, but a more direct estimate is possible using the systems developed and described in this thesis. It allows for the possibility that different membrane systems may respond in different ways to a particular group of solvents.

We measure the partition coefficient of the solvents into the cell as a whole. In this system the luciferase will report on the concentration in the aqueous phase, and the cytochrome P450 itself will report on the membrane phase. Since we keep the membrane volumes constant we were able to compare the effect of a group of solvents on each enzyme.

11.4 Profile of Inhibition

One aim of the thesis was to look for evidence of the idea that there is a critical solvent concentration in the membrane at which the enzyme is inhibited, and to propose this as the primary effect in the link between Log P and the inhibition of enzyme activity.

Using the method developed to determine the amount of solvent associated with membranes, the relationship between the inhibition of two P450 enzymes with the amount of solvent in the aqueous phase has been investigated. The partitioning of alcohols into the membrane phase has been estimated directly rather than indirectly from partition coefficient data obtained in model systems (Log Poct).

This is demonstrated by the inhibition of P450 11α from *R. stolonifer* by a homologous series of alcohols. Methanol, the first of the series has a high solubility in the aqueous phase (in practice it is completely miscible). Methanol also has a low partition coefficient between a membrane and aqueous phase. As the homologous series is ascended the solubility of the alcohol in the aqueous phase decreases and the partition coefficient increases.

The effect of the solvents as inhibitors in the reaction mixture was characterised by their IC_{50}. The soluble enzyme from *B. megaterium* was more strongly inhibited by short chain alcohols (IC_{50} for methanol = 0.71 M) than the enzyme from *R. stolonifer* (IC_{50} for methanol = 1.63 M). The inhibition of both enzymes by the primary alcohols becomes more extreme as their molecular weight increases, and they are about equally inhibited by pentanol. (IC_{50}= 62 mM & 46 mM respectively). Hexanol inhibits the enzyme from *R. nigricans* (IC_{50}=25 mM), but it is not sufficiently soluble, solubility limit in water 50.1 mM, **(Bell, 1972)** to inhibit the enzyme from *B. megaterium*.

These concentrations of solvent are the totals added to the assay mixtures. In the case *of R. stolonifer* they are therefore greater than the actual aqueous concentrations, because some solvent is taken up by the membranes of the wet cells. However over the range of alcohols studied the amount of alcohol removed from the aqueous phase reduces its concentration by about 10mM.

The evidence for the critical membrane concentration is based on the cut-off beyond which alcohols do not inhibit, and on the regular relationship between IC_{50} and C chain length. The specific membrane concentration at which a member of a solvent group inhibits enzyme activity within the membrane can be estimated from the aqueous solubility and the partition coefficient. The data in **Table 10-6** suggest that this occurs when each gram of wet cells contain about 3 mmole alcohol, but we do not know how this is distributed amongst the various membrane phases.

The concept of a critical membrane concentration does explain the sudden transition from compatible to non-compatible solvents as Log P increases because although the partition coefficients are increasing the aqueous solubilities of the solvents are decreasing even faster.

Since the ability of molecule to inhibit enzyme reaction in a two-phase system is related to the product of the solubility in the aqueous phase and the partition coefficient, the limiting factor for long chain molecules would be the solubility in the aqueous phase.
Conversely the limiting factor for short chain molecules would be the partition coefficient. As the homologous series is ascended effect increases until the solubility in the aqueous phase falls to a level that cannot support inhibition in the membrane phase. This is the membrane concentration cut off effect.

11.5 Comparison of the inhibition of P450 BM3 and P450 11α

The inhibition of purified P450 BM3 **(Figure 5-7)** and of P450 11α in whole cells of *R. stolonifer* **(Figure 7-2)** by alcohols demonstrates the importance of this difference. In each case as the chain length increases the ability to inhibit the enzyme activity increases

until a chain length is reached where no inhibition is caused. For P450 BM3 hexanol cannot reach an IC_{50} concentration whereas with P450 11α in whole cells of *R. stolonifer* hexanol can effect an IC_{50} inhibition but octanol cannot. P450 BM3 can tolerate hexanol in the system with only a 25% reduction in the activity of transformation. However the membrane bound P450 11α is inactive in the presence of this solvent. Purified P450 BM3 is therefore more resistant to alcohols than the membrane enzyme P450 11α by two carbon units. Since the substrates of many biotransformations are relatively hydrophobic, purified enzymes in this case could be used to increase the variety of organic solvents used to increase process pressures on the cytochromes P450 when used as a bioreactor.

The results demonstrate that there are differences between the two enzymes studied.
From the experimental observations we can conclude that for a homologous series of solvents the concentration of solvent bound required to cause inhibition of a fixed ratio of enzyme activity is relatively fixed. As the chain length of the inhibitor increases so too does the partition coefficient. Although we were unable to measure the partition coefficient directly, because the volume of the membrane was unknown, the results are very similar to those observed in the action of general anaesthetics. This strongly suggests that the amount of solvent per gram of cells remains relatively constant over the range of alcohols studied

11.6 Industrial Use

The rate of microbial hydroxylation of progesterone by *Aspergillus ochraceous* increases by the addition of a separate organic phase **(Ceen, 1986).** Loss of P450 11α hydroxylase activity is very rapid and almost complete within 10 minutes. This limits the industrial use of a second organic phase in large-scale biotransformations. An attempt to purify P450 11 α from whole cells resulted in loss of activity even at the homogenisation step **(Thomas, 1994).** A series of *n*-alcohols were tested for their effect on the operational stability in a two-phase system **(Osborne, 1990).** All lower alcohols tested resulted in the complete loss of activity with the exception of hexanol. *R. stolonifer* retained 50 % of the activity within 10 minutes of incubation with hexanol.

The membrane bound P450 system has been shown to be made up of four proteins: cytochrome P450, NADPH-cytochrome P450 reductase, cytochrome b5, and cytochrome b5 reductase **(Madyashtha *et al.*, 1984).** The substrate binding site of membrane bound cytochrome P450 directly faces the vertical plane of the lipid bilayer **(Taniguchi *et al.*, 1984).** The NADPH-cytochrome reductase must be continually recycled to preserve activity.

Theories have been presented to explain the deactivation of membrane bound enzymes. These include alteration of membrane fluidity, lipid phase separations, direct solvent - protein interactions, and membrane permeabilisation. Each may contribute to the overall reduction in the rate of activity of the membrane bound P450, but only direct solvent - protein interaction can explain the reduction in fatty acid hydroxylation exhibited by P450 BM3, since this occurs in the cytoplasm and in our experiments has been purified to homogeneity.

In both P450 enzymes it was noticed that at low concentrations of solvent an increase in hydroxylase activity was seen. It has been reported that benzyl alcohol at membrane concentrations of 20-100 mM caused increases in the activity of seven different membrane-bound enzymes **(Gorden *et al.*, 1980).** Membrane expansion and increased membrane fluidisation have been cited as a cause of this increased activity. An increase in membrane fluidity may allow more rapid diffusion of substrate into the membrane, resulting in higher collision frequency and higher observed rate of reaction. This explanation is not valid for P450 BM3, which is a soluble enzyme. The phemonenon has also been seen with the effect of both alkanes and alcohols on the pure bioluminescent enzyme luciferase **(Section 2).** Increased reaction rates could be a result in increased collision frequency or the stability of the transition state complex. Solvents may be beneficial in this respect.

As the solvent concentration is increased further a rapid reduction of initial activity is noted with both bound and unbound P450 enzymes. In the membrane case this may be due to a disruption of the enzyme-membrane architecture. Uncoupling of P450 activity to the solvent was examined and found not to be relevant. In the case where no membrane is

present the enzyme activity is retained at concentrations that would produce complete inactivation of the initial activity of the membrane bound enzyme. This suggests that the membrane bound enzyme the functionality is determined by the support and when the support is disrupted all activity is lost. The cytochrome itself, like P450 BM3, may be significantly more resistant to the effects of the solvents.

Some higher solvents studied produced no inactivation even at saturating concentration. This is attributed to their inability to reach a particular critical concentration in the membrane. For the range of *n*-alcohols from methanol to heptanol, the amount of alcohol per gram of membrane to cause inhibition to 50% is ~3 x 10^{-3} moles **(Table 8-2).** This concentration remains relatively fixed when compared to the calculated aqueous concentration required to cause IC_{50} inhibition (1200 mM for methanol decreasing to 6 mM for heptanol, **(Table 8-2).**

The saturated solutions of alcohols from methanol to heptanol have a sufficient concentration in the aqueous phase to partition enough molecules of solvent into the membrane to cause IC_{50} inhibition (~3 x 10^{-3} moles). Above heptanol even though the ability of this alcohol to partition into the membrane increases, the aqueous concentration is not high enough and the number of octanol molecules able to partition (determined by the true membrane / buffer partition coefficient) is not enough to exceed 3 mM/g.

It is possible that most homologous series behave in a similar way. Several of the systems for which **(Laane *et al.*, 1985)** showed the sigmoidal Log P-activity retention correlation resemble those presented in this thesis; that is, they were for microbial whole cell performing a conversion using a membrane-bound enzyme. The explanations for the observed correlation are therefore likely to be similar to those presented here. The increase in fluidity cannot explain the results with P450 BM3 or with luciferase.

To aid the selection of solvents in the future, it is necessary to determine the membrane concentrations for at least the lower members of the group. The limiting chain length relating to the cut-off effect and the solvent that allows for full activity can then be predicted and used.

Organic solvent selection can now be obtained for any two liquid-phase systems rationally. Critical membrane concentrations can be obtained in a simple and reliable manner using the stabilised form of luciferase. This allows for the elimination of a large number of solvents expected to cause complete loss of enzyme activity in industrial reactors.

11.7 Implications to anaesthesia

The critical membrane 'concentration' to cause the inhibition of membrane protein has been determined but the action on the molecular level has yet to be determined but some form of protein-membrane disruption is the favoured hypothesis from the results of this work

The work by **(Franks & Lieb, 1984)** indicated that the site of general anaesthesia could be protein based rather than the membrane theories traditionally accepted. They demonstrated how the cut off effect could be explained by the use of a membrane protein target site of anaesthetics with the use of purified luciferase. Cytochromes P450 have been suggested as possible protein target **(Franks & Lieb, 1987).** For *R. stolonifer* a direct determination of membrane IC_{50} concentrations has shown that the inhibition of a membrane protein can be effected at the same concentration of a number of alcohols of differing chain length at a single concentration at the membrane. This supports the protein target hypothesis of anaesthesia. Attempts to correlate the EC_{50} data for a range of isolated organs and animals fails to give good correlation and P450 11α seems to be effected by higher alcohols to a greater extent than for mammalian P450 systems **(Table 10-3).**

n-Alcohol	EC_{50} (mM)	Log_{10} EC_{50}	IC_{50} (mM)	Log_{10} IC_{50}
Methanol[a]	790	2.898	1	3.09691
Ethanol[b]	263	2.420	1000	3
Butanol[c]	1	1.256	200	2.30103
Pentanol[d]	2.9	0.462	50	1.69897

Table 11-1 Relationship between anaesthetic potency (EC50) and potency of inhibition for inhibition of progesterone metabolism (IC_{50}).

EC_{50} values are derived from studies on isolated organs, aquatic organisms, and animals. References for each value are as follows (cf. Superscripts in the table): *a*, 590 (Alifimoff *et al* 1989), 990 (Vernon, 1913); *b*, 190 (Alifimoff *et al* 1989); 410 (Vernon, 1913); 330 (Meyer and Hemmi 1935), 120 (Pringle *et al.* 1981); *c*, 10.8 (Alifimoff *et al* 1989), 22.3 (Vernon, 1913), 30 (Meyer and Hemmi 1935), 12.0 (Pringle *et al.* 1981); 17.0 (Paton 1974); 13.4 (Elliott and McElwee 1988); *d*, 2.9 (Alifimoff *et al* 1989), 3.2 (Elliott and McElwee 1988); 2.5 (Roth 1980).

11.8 Implications to industrial solvent issues

Simple tests have been developed to determine the maximum chain length of a homologous series that would support a biotransformation in the aqueous solvent mixture. Given that we know the range of solvents that can be utilised what chain length should be used and what homologous series should be chosen? These choices would be affected by the regulatory affairs, solvent formulation, health and recovery issues.

11.8.1 Introduction

Industrial solvents are generally liquid organic compound used on a large scale. Compared with the large amount of potential liquid organic compounds available, economic, safety, manufacturing and environmental issues limit scope to a few chemical classes. Aromatic

and aliphatic hydrocarbons, alcohols, ketones, esters, glycols, glycol ethers, chlorinated hydrocarbons, amines and aldehydes.
The selection of a suitable solvent and blend development is a combination of the issues raised in the previous paragraph.

11.8.2 Regulatory affairs

Since 1966, new and more restrictive regulations have been adopted by the controlling bodies in both the European Economic Community (ECC) and the numerous organisations in the USA. Regulations have been a driving force in the development of new technologies and products that decrease solvent emissions to minimise environmental impact.
European and US air quality standards have been set for ozone levels and although solvent operations do not emit ozone directly, solvents, as volatile organic compounds (VOCs), react with nitrogen oxides in the atmosphere to produce photochemical smog, of which ozone is a significant component.
Different regulatory approaches have been adopted that lower total VOC levels and restrict the range of allowable compounds to those that have a low photochemical reactivity and so give a smaller contribution to the smog levels.
The clean air act, as amended in 1990 represent a more recent effort by the US congress to address clean air concerns. A compliance timetable by category as been established, which depends on the level of current ozone concentration. The definition of a major source also depends on the category of ozone non-attainment **(Table 11-2)**. The reduction of hazardous air-pollutants also feature in the amendments and is regulated by the Environmental Protection Agency (EPA). The EPA insist in the installation of maximum achievable control technology (MACT) and once MACT controls have been met further control is applied to reduce residual emissions.

It is clear that the use of organic compound in the chemical industry is under ever more strict regulation and that the range of compound that can be used is decreasing rapidly.
There is a constant drive to remove all organic compounds out of the manufacturing process. The use of free enzymes or whole cell bio-reactors in modern large scale bio-

processing will ultimately be a trade of between safety and cost-benefit of using such technology.

Classification	Deadline to attain	Major VOC source, t/ty
Marginal	Nov. 15, 1993	100
Moderate	Nov. 15, 1996	100
Serious	Nov. 15, 1999	50
Severe	Nov. 15, 2005 to Nov. 2007	25
Extreme	Nov. 15, 2010	12

Table 11-2 VOC Emission is Reduction/Ozone Attainment Schedule

11.9 Solvent Recovery

The recovery of valuable solvents is an important factor in determining the overall economics of a chemical and biochemical large-scale process. Processes employing solvents have generally included solvent-recovery systems. Solvent recovery issues relating to MACT and the cost of solvents difficult to produce have directed the development of complex solutions to the problem.

In our hypothetical bio-reactor the chosen solvent is recycled after the separation of substrates, products, and possibly enzyme depending on cost.

Efficient solvent recovery will result in low solvent use, and therefore low activity in the manufacture of chemicals specifically for solvent applications.

11.9.1 Solvent-Recovery Systems

Many solvent-recovery operations are based on the same techniques, such as absorption, adsorption, extraction, filtration, distillation, and condensation. The same factors such as volatility, solubility, thermal stability, corrosion, purity requirements, capacity, steam and water conditions, safety, and economics must be taken into account.

11.9.2 Solvent -Recovery Techniques

11.9.2.1 Mechanical Separation

Draining of liquids from solids is a common operation in solvent processes. The solids are usually retained either by stationary or moving screens by some form of agitation. Centrifugal filters and solid bowl centrifuges use centrifugal force to increase the efficiency of filtering but lead to an increase demand for power. This process is best suited for product recovery systems where the substrate can be recycled without loss or with a low cost substrate (relative to product).

11.9.2.2 Extraction

Solvent and product are sometimes separated by washing with water or another solvent **(Robbins, 1979).**

Low viscosity solvents can be extracted with and aqueous wash. If the solvents are viscose however an emulsion may result giving rise to low extraction efficiencies.

pH of the solution may be altered to alter the concentration of product in the aqueous or water phase. For example a change in pH from 7.5 to 2.5 converts penicillin form a preferentially water-soluble compound to a preferentially organic soluble one.

11.9.2.3 Evaporation

Simple evaporation and condensation can recover organic solvents. The cost of evaporating solvent increases with the chain length and for higher chain length compounds that are solid at room temperature, elevation of pressure or temperature would have to be employed increasing the cost of the process.

11.9.2.4 Other processes

These include fractional distillation, drying in the absence of air or inert gas, drying with air or inert gas, condensation, absorption, and adsorption.

11.10 Process Economics

Solvent-recovery decisions are based mainly ion economic analysis. Such analysis involves not only careful engineering calculation, but also a prediction of future prices of raw materials, utilities, and products, of interest rates on borrowed capital, tax rates, and of government standards and regulations with regard to pollution and occupational safety. Methanol, for instance, is easily dehydrated by straight fractional distillation. Ethanol and propanol form constant -boiling mixtures with water. Butanol is easily dehydrated since the two layers can be decanted and stripped. When purification of the recovered solvent is difficult, a less pure solvent might be acceptable for recycling.

11.11 Health and Safety Factors

Safety considerations may ultimately form the basis for choosing a particular solvent and determine the recovery process. Organic solvents not only pose a flammable threat but also the solvent vapours can be neurotoxic, for example in the case of long chain alcohols. Increased interest in the long-range effect on human health exposure to solvents and other chemicals have led to the development of 'cradle-to-grave stewardship' over the chemicals they used or produce, monitoring both their own and their customers' safety precautions. The effect of these developments on solvent-recovery and the choice of organic solvents will, of course, be an increased preference for innocuous solvents and for systems giving essentially complete recovery.

12. Future work

12.1 The solvent assay

The production of substrate-stabilised enzyme could be developed to further improve the stability. This would have implications on storage and cost if the detection kit was used in a large scale.

To modify the purification procedure, reducing the number of steps. Isolation of P450 BM3 from *E. coli* would give benefits to the procedure and could yield higher process efficiency.

To estimate the uptake of solvents into the cells, under a wider range of conditions than was possible in the present experiments. These would include the effect of cellular age and growth, the effect of temperature and the effect of cell concentration. The latter should improve the accuracy of the estimate of solvent uptake.

12.2 Testing other Solvents

Although both n-alkanes and n-alcohols were used to develop and test the luciferase assay, only n-alcohols were tested with the soluble and membrane bound Cytochromes because of time constraints. Investigating n-alkanes would give a good opportunity to test if the molecular cut off effect was apparent in other chemical groups.

Determining the effect of other *n*-alcohols with the same Poct value on the activity of both hydroxylases would yield information relating the effect of molecular shape on the inhibition. The comparison of the two enzymes should extend through a much wider range of solvents.

Immobilisation of whole cell membrane fragments on to a hydrophobic resin could be

investigated to discover if this had any effect on the inhibition.

12.3 Engineered Enzymes

In our original experiments we were not able to obtain a membrane bound enzyme that would report on the concentration of solvent in the membrane phase with a soluble version of the same enzyme acting as a control. Genetic engineering gives the biochemist the tools to change the structure and function of many enzymes. Genetic engineering may yield a membrane free mutant of P450 11α. We could test the wild form with the mutant as the control enzyme. In the future different Cytochromes may be expressed in the cellular membranes of other species. Once this has been achieved, using the tools developed in this thesis it would be possible to test a variety of situations not available to us, and to move the same enzyme in different cellular locations. We could test the partitioning of solvents into a range of membranes and determine if the structure of these different membranes affects the partitioning of solvent.

Genetic engineering may also be able to produce an over-expressed membrane bound Cytochrome P450. Since the proportion of membrane containing P450 would be higher than the wild form a test could be developed to determine if the different membrane types within a species have different partition characteristics.

13. References

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